PHOSPHORYL TRANSFER REACTIONS CATALYSED BY FE(II).

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Icenius de Zwart

University of Canterbury
Christchurch
New Zealand

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Abstract.

This thesis probes the catalysis of phosphoryl transfer reactions by Fe(II), with particular emphasis on biologically relevant examples. The results of this thesis may have important implications for the origin of metabolism and the origin of phosphate in metabolism.

Chapters two and three describe the phosphoryl transfer reactions of acetyl phosphate and phosphoenolpyruvate in the presence of Fe(II). The effect of various factors, including the pH, amount of Fe(II) present and temperature are investigated. Fe(II) was found to be an effective catalyst for phosphoryl transfer to inorganic phosphate. Phosphoryl transfer from either acetyl phosphate or phosphoenolpyruvate to inorganic phosphate may provide a feasible route for the prebiotic production of pyrophosphate.

The hydrolysis of polyphosphates is described in chapter four. Pyrophosphate was found to be more stable with respect towards hydrolysis in the presence of Fe(II) compared to in the absence of Fe(II). Tripolyphosphate, ADP and ATP were found to be less stable with respect to hydrolysis in the presence of Fe(II). The hydrolysis of ATP in the presence of Fe(II) was found to proceed by two pathways. The major pathway involved loss of the terminal phosphate and resulted in the formation of ADP and phosphate. However, a significant amount of ATP hydrolysed by an alternative pathway that resulted in the production of AMP and pyrophosphate. The effect of Fe(II) on the hydrolysis of these compounds may have implications for the selection of ATP as the predominant source of free energy in biological systems.

Abbreviations.

2-PG	2-phosphoglycerate
3-PG	3-phosphoglycerate
AcP_i	acetyl phosphate
acetyl CoA	acetyl coenzyme A
A	adenine
Ad	adenosine
ADP	adenosine 5′-diphosphate
AMP	adenosine 5′-monophosphate
ATP	adenosine 5′-triphosphate
BSA	bovine serum albumin
CoASH	coenzyme A
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Ga	billion years ago
GC-MS	gas chromatography-mass spectroscopy
GDP	guanosine 5′-diphosphate
GTP	guanosine 5′-triphosphate
HEPES	4-(*N*-2-hydroxylethyl)-1-piperazine-*N*-2′-ethanesulphonic acid
kJ/mol	kilojoules per mole
M	moles per litre
MES	4-(*N*-morpholino)ethanesulphonic acid
mM	millimoles per litre
MOPS	3-(*N*-morpholino)propanesulphonic acid

NAD^+	nicotine adenine dinucleotide (oxidised form)
NADH	nicotine adenine dinucleotide (reduced form)
NDP	nucleotide 5′-diphosphate
NMR	nuclear magnetic resonance
NTP	nucleotide 5′-triphosphate
Nu	nucleophile
PEP	phosphoenolpyruvate
P_i	inorganic phosphate
PIPES	1,4-piperazine-*N*,*N*′-bis(2-ethanesulphonic acid)
PP_i	inorganic pyrophosphate
PPP_i	inorganic tripolyphosphate
RNA	ribonucleic acid
Tris.HCl	tris(hydroxymethyl)aminomethane hydrochloride
tRNA	transfer RNA
UV	ultraviolet
μL	microlitres

We must, however, acknowledge, as it seems to me, that man with all his noble qualities...still bears in his bodily frame the indelible stamp of his lowly origin.

Charles Darwin

Real knowledge is to know the extent of one's ignorance.

Confucius

1 Introduction

1.1 The Origin of Life.

The question of the origin of life on earth has puzzled mankind for millennia. There are many theories regarding the origin of life, spanning the spectrum from Divine intervention to blind luck. However, many of these theories can not be tested scientifically and it is only in the last century that scientific methodology has been brought to bear on the problem. A number of scientific disciplines, including geology, chemistry and the biological sciences, have contributed to current theories regarding the origin of life.

The origin of life is intimately associated with the origin of metabolism, the chemical reactions that are carried out in every living cell. To begin to develop an understanding of the origin of life, we first have to find out where life arose, when life arose and what life arose.

1.2 Where?

There are two potential places where life could have arisen; on earth, or somewhere else. Since life exists on earth, and has only been found on earth, the simplest assumption is that life arose here. However, a number of people have advocated that life arose somewhere else in the universe, and came to earth via meteorites or comets. They argue that life is too complex to have arisen in the time available for it on earth.[1]

Assuming that life arose on earth, it is worthwhile finding out under what conditions life arose. Geochemistry has provided information about the early earth and the types of environments that life may have arisen in.

1.2.1 The environment of the early earth.

Darwin, in a letter from 1871 speculated on the origin of life.[2]

> *It is often said that all the conditions for the first production of a living organism are now present, which could ever have been present. But if (and oh! What a big if!) we could conceive in some warm little pond, with all sorts of ammonia, phosphoric salts, light, heat, electricity, etc., present, that a proteine compound was chemically formed ready to undergo still more complex changes, at the present day such matter would be instantly devoured or absorbed, which would not have been the case before living creatures were formed.*

However, the conditions on the early earth were probably far removed from Darwin's 'warm, little pond'. Dante's seventh hell may be a more apt description.

1.2.2 The atmosphere.

The atmosphere of the early earth has been the subject of much debate. It was originally thought to be highly reducing, containing large amounts of methane, ammonia, hydrogen and, possibly, carbon monoxide. Now, however, it is thought to have been composed predominantly of carbon dioxide, nitrogen and water, with small amounts of methane, hydrogen sulfide, and hydrogen.[3] There was very little oxygen present, as what little was formed would have reacted almost immediately with hydrogen or metals. Because of the lack of an ozone layer, UV radiation would have been extremely harsh and would have prevented the build-up of organic compounds near the surface of the oceans. This is because the energy of UV radiation is of the same order of magnitude as the energy of the bonds in organic molecules and results in the breaking of those bonds. UV radiation also penetrates water to a depth of a few metres.

1.2.3 The oceans.

Liquid water is thought to have existed on earth by about 4.3 billion years ago[4]. The temperature of the ocean is a contentious issue and almost any temperature from 0-150°C can be supported.[5] In all likelihood it was probably quite warm, around 50-80°C, due to a combination of radioactive decay, geothermal activity, meteorite impact and the greenhouse effect. In any case there would always have been hot environments associated with hydrothermal activity. The ocean was slightly acidic due to dissolved carbon dioxide, hydrogen chloride and sulfur dioxide. The redox potential of the ocean was reducing due to the presence of sulfides and hydrogen. The ocean may also have contained a high concentration of metal ions such as Na^+, K^+, Mg^{2+}, Ca^{2+} and Fe^{2+}. The seafloor was most likely very active, with large cracks between the continental shelves, and a high level of volcanic activity. These processes would have ejected huge amounts of superheated acidic water, laden with minerals and hydrogen sulfide. The ocean may have been almost black due to the presence of vast quantities of metal-sulfide precipitates. These areas are potentially very interesting as sites for the origins of life because of the large flow of energy and material that occur there **(Figure 1.1)**.[6] Similar vents have been found in present day oceans where the seafloor is spreading.[7] These vents are called black smokers due to the presence of large plumes of metal-sulfide precipitates and harbour large communities of organisms, who derive all their energy requirements from the vent system.

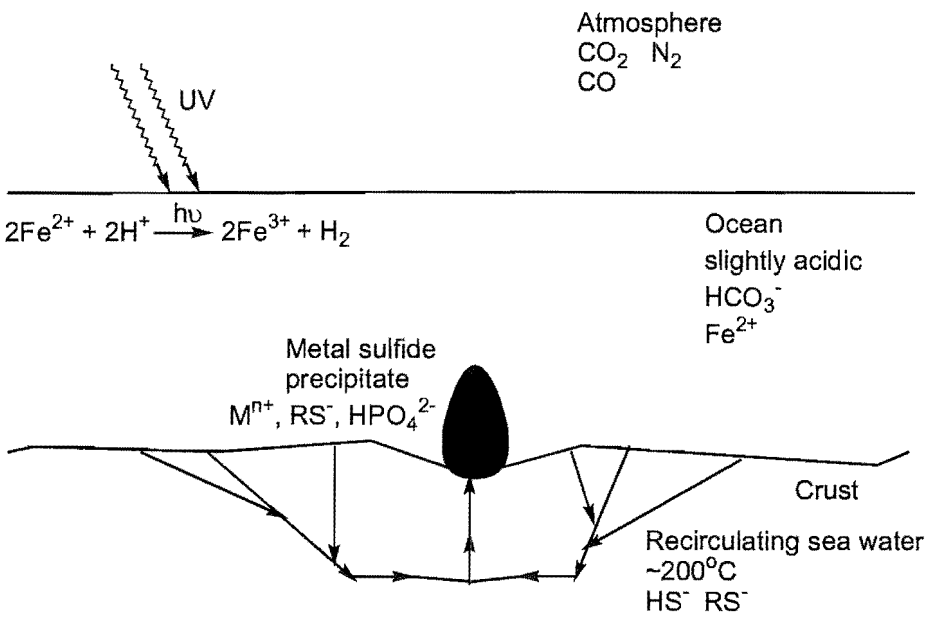

Figure 1.1. A possible environment for the origin of life on the early earth.

1.3 When?

Answering the question "when did life arise?" requires knowledge about the history of the earth and of life. Two lines of evidence have been used to determine upper and lower limits for the time of the origin of life. The formation of earth provides an upper limit on the possible age of life. Looking back through the fossil record and for geological evidence for life allows a lower estimate for how long life has existed on earth.

Information about the history of earth has been gleaned from radioisotope data and from geology. Radioisotope data suggests that the solar system began to form about 5 billion years ago (Ga), with the aggregation of the planets occurring over the next 500 million years.[8] The earth at this stage would have been a largely molten ball of magma. This would have slowly cooled, with a crust forming and water condensing to form the oceans over a time period of a few hundred million years. Meteorite impacts were also quite frequent during this time period, with the earth experiencing a number of large impacts that vaporised any oceans and sterilised the planet.[9] This

meteorite activity is thought to have subsided about 4 Ga, and this currently is believed to be the earliest that life may have arisen.

The earliest estimates for the presence of life on earth come from isotope data in rocks from Greenland dated at 3.8 billion years old.[10,11] Microfossils that resemble prokaryotic cells were also found. Organic carbon enriched in ^{12}C as opposed to the heavier ^{13}C is considered evidence of biological activity, since in catalytic systems the kinetic isotope effect leads to the enrichment of the lighter isotope.[3] This is also true for the enrichment of ^{32}S compared to ^{34}S. Evidence of microbial communities in the form of stromatolites has been found in 3.5 billion-year-old rocks from Australia.[12] These fossils are very similar in morphology to present-day cyanobacteria. Based on this geological evidence, life is assumed to have arisen some time between about 4 and 3.8 billion years ago.

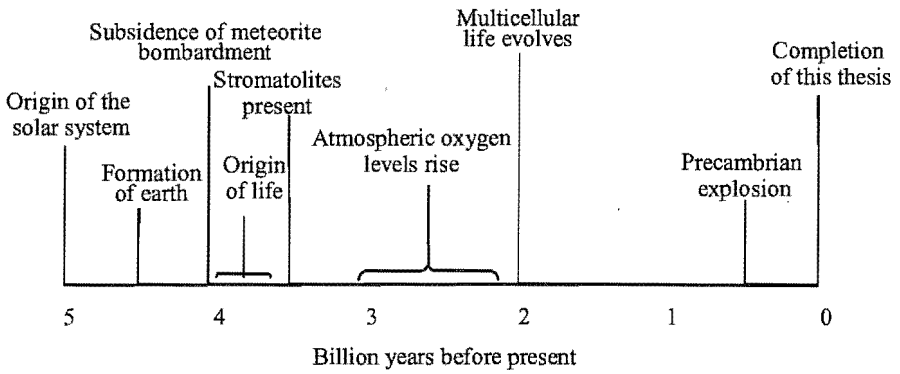

Figure 1.2. Timeline indicating major events in the history of the earth.

1.4 What Was Life Like?

The earliest life forms are likely to have been vastly different from modern-day organisms; they would have been much simpler than the simplest organism that we know of. As stated in the previous section, fossils have been found in very ancient rocks and provide an indication of the time frame of evolution. Unfortunately, these fossils do not tell us much about what the first organisms were like. Instead, recent

advances in biochemistry and molecular biology have allowed educated guesses to be made, based on present-day organisms.

The technique of comparative sequencing of biological macromolecules, such as DNA and proteins, has allowed the evolutionary relationships between organisms to be probed.[13] This technique is based on the assumption that sequence similarities in genes from different organisms reflect a common ancestry, an assumption that has been verified many times. Comparisons of the sequences between many organisms allows a phylogenetic tree to be constructed, in which the distance between any two organisms from their common ancestor reflects the number of changes that had to have occurred to account for the differences in their gene sequences. Although the finer details about the 'tree of life' are still being hotly debated,[14] the overall topology of the tree is generally agreed upon, with present day organisms being divided into three kingdoms: eubacteria; archaea; and eucarya (see figure 1.3). The root of the tree represents the last common ancestor of life. The position of the root of the tree is the subject of much debate, but it is believed to lie between the branches leading to the eubacteria and the archaea. When the last common ancestor lived is also uncertain. The existence of 3.5 billion-year-old stromatolites suggests the presence of photosynthetic bacteria, which in turn implies that the first branching of the tree had occurred. This means that the last common ancestor existed over 3.5 billion years ago, not long after the origin of life.

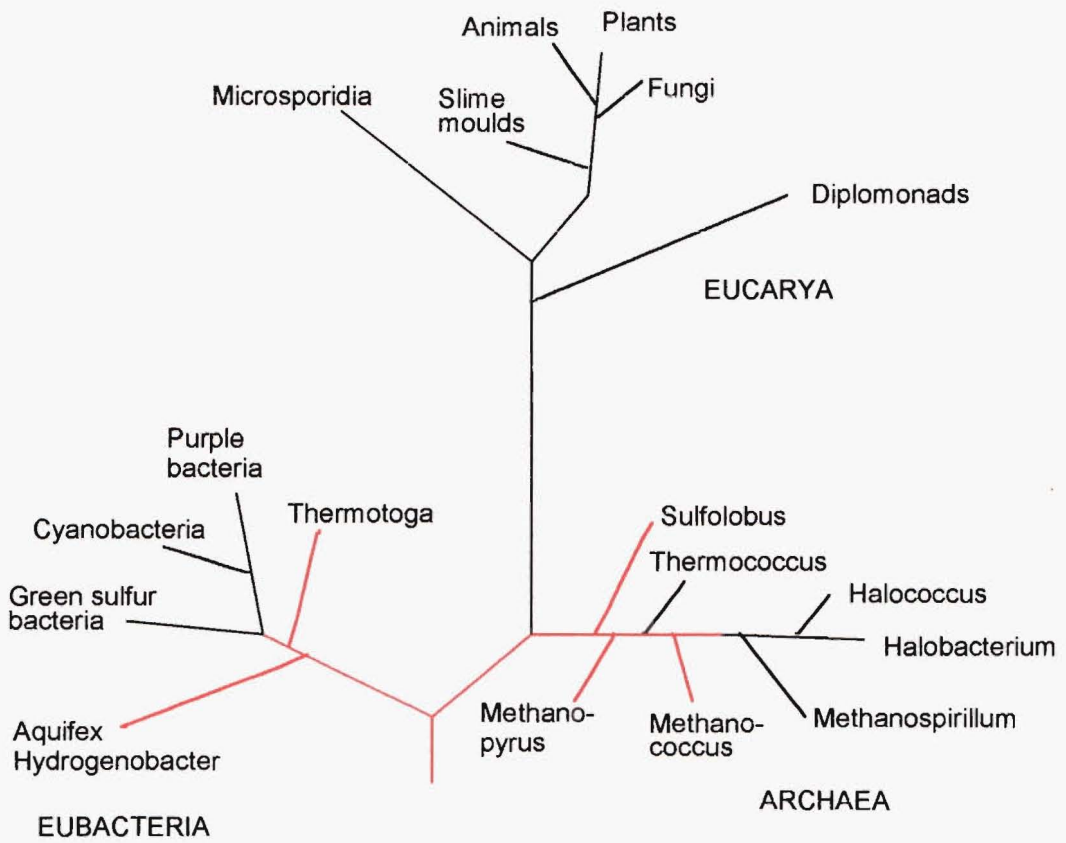

Figure 1.3. Tree of life showing that the oldest lineages (in red) are thermophilic.[15]

The properties of the earliest organisms may be inferred from the properties of present-day organisms that lie closest to the base of the tree **(Figure 1.3)**. These organisms have undergone the least amount of change and hence may have retained more of the characteristics of the common ancestor than organisms that have evolved more. The most deeply branching lineages of both Eubacteria and Archaea are all thermophilic, and most have a metabolism based on chemoautotrophy (where inorganic compounds provide all the energy and nutritional requirements of the organism). This has lead to the suggestion that the origin of life occurred in a geothermal setting, and that the first organisms were autotrophic. This assumption is not universally accepted, and opponents argue that the last common ancestor was already quite sophisticated and may have evolved from organisms that had very different properties.[16]

Since modern organisms have evolved from earlier ones it is likely that they may have retained some of the features found in the first organisms. The features that are most likely to have been conserved are those features that are essential for all life. All known life-forms share some essential features, and these include: a lipid membrane encapsulating the cell; proteins for structural and catalytic roles; and nucleic acids as a store of genetic information.

Certain aspects of metabolism are also conserved throughout life and these may have been present in the earliest organisms. These include core metabolic processes, such as the citric acid cycle and glycolysis, which, in some form, are found in all organisms. The use of polyphosphates and thioesters to drive chemical reactions is also ubiquitous. Understanding how these metabolic processes evolved may be one of the keys to understanding the origin of life.

1.5 Theories Regarding the Origin of Life.

Life requires a number of essential compounds, such as amino acids and sugars, which must have been available for life to begin. All organisms can be divided into two categories, heterotrophs or autotrophs, depending on how they obtain these compounds. Heterotrophs are organisms that use organic compounds, such as sugars, as their source of energy and building blocks. These organisms include animals, yeasts and many familiar bacteria such as *E. coli*. The autotrophs are able to gain all of their energy and their nutritional requirements from inorganic sources, such as carbon dioxide and light. Plants and algae are familiar examples, and there are also a large number of microorganisms that are autotrophic. Theories regarding the origin of life can be classified according to these two groups, depending on whether the first organisms were heterotrophic or autotrophic.

1.5.1 Heterotrophic origin of life.

The heterotroph-first theory has been the dominant theory on the origin of life for the last century, since Darwin first speculated about some 'warm little pond'. In the model developed by Oparin[17] in the 1920's, a primitive ocean accumulated a large variety of chemicals that were produced on the early earth through various chemical processes. In this way the ocean became a 'soup' of energy-rich biochemicals, within which arose the first primitive organisms. Because of the presence of a variety of nutrients, these organisms did not have to synthesise any of the compounds of primary metabolism, the amino acids, nucleotides and other metabolites, essential for life. Only when the supply of nutrients began to run out did a need arise for primary metabolism, at which point these processes evolved.

A heterotrophic model for the origin of life requires that all the molecules required for life, such as amino acids and sugars, be present in some form to sustain a heterotrophic community. This requires a source of organic compounds.

Extraterrestial formation of organic compounds.

Many molecules have been identified spectroscopically in interstellar space, some of which may have relevance to the origin of life. These include simple molecules such as CO and HCN, as well as more complex organic molecules. Some of these molecules are listed below **(Figure 1.4)**.

H_2	CO	HCCCN
HCN	H_2O	H_2CCHCN
NH_3	CH_2O	H_2CNH
CH_4	HCO_2H	H_3CCN

Figure 1.4. Molecules of relevance to the origin of life identified in space[18].

A large amount of organic material may have been delivered to the earth by meteorites, particularly early in the history of the earth when meteorite bombardment was quite heavy.[19] Meteorites often contain up to a few percent by weight of carbon, in a variety of different compounds, including hydrocarbons, carboxylic acids, amino acids and aldehydes. Among the meteorites that have been found on earth, the Murchison meteorite, a carbonaceous chondrite that fell in Australia in 1969, is worth mentioning. This contained a large amount of carbon, including a number of amino acids **(Figure 1.5)**.

Glycine	Alanine	α-Aminobutyric acid
Valine	Norvaline	α-Aminoisobutyric acid
Isovaline	Proline	β-Aminobutyric acid
Pipecolic acid	Aspartic acid	β-Aminoisobutyric acid
Glutamic acid	β-Alanine	γ-Aminobutyric acid
Sarcosine	*N*-Ethylglycine	*N*-Methylalanine

Figure 1.5. Amino acids identified in the Murchison meteorite.[20]

Terrestrial formation of organic compounds.

Another source of organic material is through prebiotic synthesis from inorganic starting materials. The first experiments conducted in this area where those of Miller in 1953, who passed electrical discharges, to mimic lightning, through an atmosphere of methane, water, ammonia and hydrogen, the presumed atmosphere of the primitive earth.[21] After a few days a wide variety of organic compounds had been produced, including many amino acids **(Figure 1.6)**.

Compound	Yield (% of CH_4)
Formic acid	4.0
Glycine	2.1
Glycolic acid	1.9
Alanine	1.7
Lactic acid	1.6
β-Alanine	0.76
Proponoic acid	0.66
Acetic acid	0.51
Iminodiacetic acid	0.37
α-Aminobutyric acid	0.34
α-Hydroxybutyric acid	0.34
Succinic acid	0.27
Others	0.62

Figure 1.6. Compounds formed from sparking $CH_4 + NH_3 + H_2O + H_2$.

However, current models predict that a much less reducing atmosphere was present, consisting mainly of carbon dioxide, nitrogen and water, with small amounts of methane, ammonia and carbon monoxide.[3] Under these conditions the yield of organic compounds in Miller-type experiments is greatly reduced.

Many of the biological monomers required for life have been synthesised under various "prebiotic conditions". These include amino acids by the Strecker synthesis **(Figure 1.7)**,[22] the bases of DNA and RNA by the polymerisation of cyanide[23,24] **(Figure 1.8)**, and sugars via the formose reaction **(Figure 1.9)**.[22]

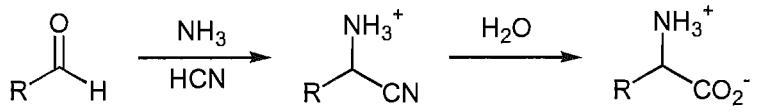

Figure 1.7. The Strecker synthesis of amino acids.

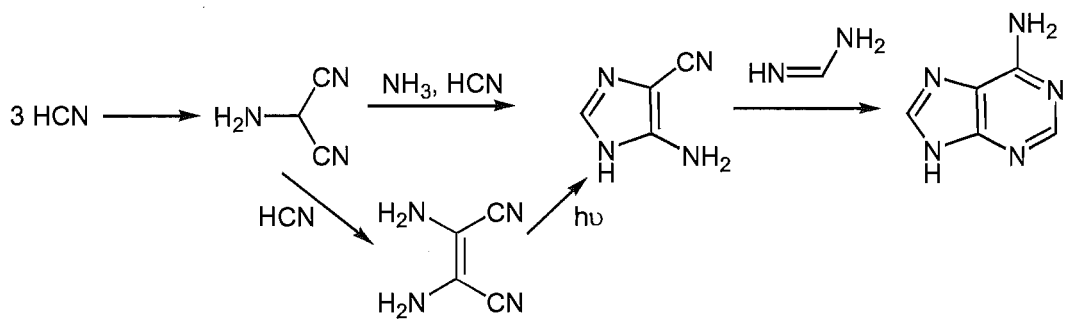

Figure 1.8. Formation of adenine by the polymerisation of HCN.

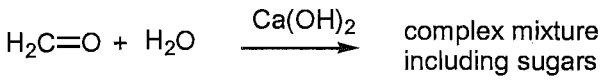

Figure 1.9. Formose reaction.

The formose reaction typically leads to a complex mixture of sugars in which the sugar unit of RNA, ribose, was identified as a minor component. In a remarkable experiment,[25] Eschenmoser and colleagues showed how a minor modification, the addition of a phosphate group, leads to a far less complex reaction mixture, with racemic ribose-2,4-diphosphate being the major component (33%) **(Figure 1.10)**.

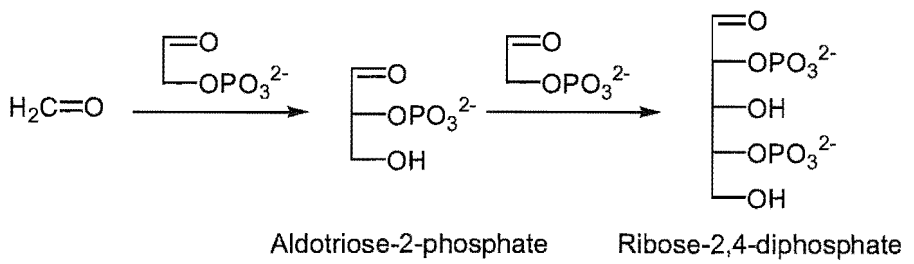

Figure 1.10. Modified formose synthesis of ribose-2,4-diphosphate

It is likely that a large number of organic compounds were present on the early earth. Whether or not they were present in sufficient quantity to give rise to, and support, living systems is debatable. The main problem with the heterotrophic theory for the origin of life is the rather large gap that exists between a solution of organic chemicals and a sophisticated entity capable of Darwinian evolution.

1.5.2 Autotrophic origin of life

The alternative theory is an autotrophic one, where the first organisms obtained all of their requirements from inorganic sources. An autotrophic theory requires the first organism to be based on metabolic cycles that could fix carbon, from carbon dioxide or carbon monoxide, into organic compounds. Of all the characteristics that are used to define a living system only a metabolism is regarded as being absolutely essential. In effect, autotrophic theories envisage complexity arising through an expanding metabolism, in contrast to heterotrophic theories, which conceive a metabolism converging out of complexity.

An autotrophic origin of life requires the development of chemistry to assimilate inorganic compounds, such as carbon dioxide and nitrogen, into organic compounds. This chemistry may mimic the metabolic pathways for biological carbon fixation and nitrogen fixation found in extant organisms. In this scenario, life would invent autotrophic metabolism right from the start, rather than having to develop it later on

when the 'soup' began to run out. The synthesis of the biological monomers, such as sugars, amino acids and fats, from inorganic precursors (e.g. CO_2, NH_3, H_2O) requires a considerable amount of reduction **(Figure 1.11)**. Reductive chemistry requires a source of electrons and since electrons do not exist as separate entities an electron donor is needed. Two possible sources of electrons have been postulated recently, both centred on ferrous iron; the photooxidation of Fe^{2+} and the oxidation of ferrous sulfide.[26, 27]

$$6CO_2 + 24H^+ + 24e^- \longrightarrow \underset{\text{glucose}}{C_6H_{12}O_6} + 6H_2O$$

$$16CO_2 + 92H^+ + 92e^- \longrightarrow \underset{\text{palmitic acid}}{C_{15}H_{31}CO_2H} + 30H_2O$$

Figure 1.11. Reactions highlighting the importance of reduction for autotrophy

The photooxidation of Fe^{2+} by UV light produces hydrogen and Fe^{3+} in aqueous solution **(Figure 1.12)**. UV light provides the energy required to raise the electrons to a high enough energy level so that they can reduce hydrogen ions. In this instance, hydrogen ions are the electron acceptors, although other molecules may also accept electrons. This process has been suggested as an alternative explanation to oxidation by molecular oxygen for the occurrence of banded iron formations, iron rich deposits containing up to 60% ferric iron.[28] These deposits have been found all over the world with ages ranging from 1.5 billion years to 3.8 billion years old, and are due to the precipitation of insoluble ferric and ferrous oxides.

$$2Fe^{2+} + 2H^+ \xrightarrow{h\upsilon} 2Fe^{3+} + H_2$$

Figure 1.12. Photooxidation of Fe^{2+}.

An alternative source of reducing equivalents may have been the oxidation of ferrous sulfide to form pyrite **(Figure 1.13)**. The driving force this reaction comes from the

stability of pyrite. The FeS/H_2S system has been shown to reduce a variety of organic compounds, including phenylpyruvate to phenylpropionate, and mercaptoacetate to acetate.[29]

$$FeS + H_2S \longrightarrow FeS_2 + H_2$$

$$2FeS + 2H^+ \longrightarrow FeS_2 + Fe^{2+} + H_2$$

Figure 1.13. Pyrite formation from the oxidation of ferrous sulfide.

Two interesting models based on an evolving metabolism that have been developed recently are those of de Duve [27] and of Wächtershäuser.[26] De Duve's theory makes extensive use of iron and sulfur, with the photooxidation of Fe^{2+} as a source of reducing power and thioesters playing a central role as a source of energy. Wächtershäuser postulates a metabolism based on surface chemistry, with ferrous sulfides acting as a catalyst and an energy source, through the reduction of FeS to pyrite (FeS_2). Thioesters also play an important role in Wächtershäuser's theory, being intermediates in carbon dioxide fixation and in amide bond formation. Wächtershäuser has provided some compelling experimental evidence to support his theory, including the reductive amination of α-ketoacids to produce amino acids[30] **(Figure 1.14)** and carbon fixation via a pathway that mimics the biochemical formation of acetyl CoA[31] **(Figure 1.15)**.

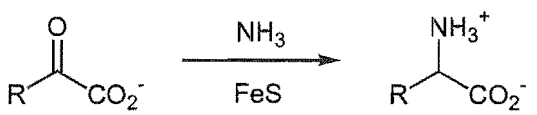

Figure 1.14. Reductive amination of α-ketoacids.

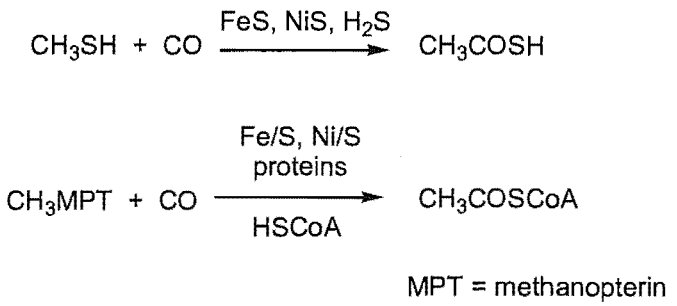

Figure 1.15. Biomimetic carbon fixation mediated by Fe/Ni sulfides (top) and carbon fixation in methanogens (bottom).

A qualitative difference between the two theories is that Wächtershäuser's requires no abiotic production of organic compounds, while de Duve sees abiotically produced organic compounds, such as carboxylic acids, as primers for subsequent metabolic cycles.

However life may have evolved, it has always required catalysts and a source of energy. Over the roughly four billion years in which life has had to evolve, nature's catalysts, and the ways in which life harnesses energy, have become increasingly complex and efficient. However, features of the original catalysts and energy-harnessing processes may have been retained. For this reason we must have a closer look at the catalysts that nature uses, and the means by which energy is harnessed.

1.6 Life Requires Catalysts.

Living systems carry out hundreds of reactions simultaneously under very mild conditions in water (typically 1atm pressure, pH ~ 7, 30-40°C). These reactions require catalysts in order that they proceed at a reasonable rate, and to ensure specificity. Catalysts are required because most biological molecules are kinetically stable, and to ensure that one reaction pathway is favoured over others, since most biomolecules can react in a number of different ways. The biological catalysts today are usually enzymes, proteins that catalyse a specific reaction. The enzymes provide binding sites suitable for the natural substrates or close analogues only; the bound

substrates are orientated in such a way that they interact with groups in the active site that catalyse the desired reaction. Often they utilise cofactors, such as metal ions or small organic molecules, to carry out a chemical transformation. Examples include iron-sulfur clusters for electron transfer, metal ions for phosphoryl transfer and coenzyme A for acyl transfer.

A primitive metabolism also required catalysts, particularly to activate the small inorganic molecules, such as carbon dioxide and nitrogen, needed to synthesise the monomers of life; the sugars, amino acids, fats and nucleotides. The activation of small molecules was probably carried out by metal ions, such as Fe, Mn, Zn, Ni, Co and Mo, and the non-metals S and Se, and these are often still found in enzymes carrying out the same reactions today. For example, the ubiquitous nature of iron-sulfur clusters may be a result of an earlier dependence on iron sulfide as a catalyst. Some of the products of a primitive metabolism may also have been able to act as catalysts in association with metal ions. These may have led to the cofactors that are required for many biochemical transformations.

An important point to realise about enzymatic catalysis is that enzymes do not invent new chemistry, instead '*enzymes ratify organic chemistry*'.[32] They can only utilise the chemistry that is inherent in the substrate. Most biological compounds are able to react in a number of different ways, and at various functional groups. Enzymes are just extremely good at accelerating one reaction pathway, and not affecting others. In order to understand how enzymes work we need to know the chemistry inherent in the substrate and in the enzyme active site. In addition, knowledge of this intrinsic chemistry may provide insights into why certain chemicals, and not others, were chosen during the evolution of life.

1.7 Life Requires Energy.

Living systems are characterised by a state of homeostasis; they are able to maintain a relatively stable internal environment even though they constantly exchange material with a comparatively unstable external environment.[33] This homeostatic state is also far removed from equilibrium and therefore organisms require a constant source of energy to maintain their viability. This includes the ability to carry out biosynthetic reactions, perform mechanical work, transport chemicals against concentration gradients, energise electrons and generate heat. Today organisms are able to meet their energy requirements in a number of ways. Energy can be obtained directly from the environment, from the sun or from reduced inorganic compounds, or indirectly by metabolising organic compounds.

1.7.1 Energy conserving reactions

The ultimate source of energy for life comes from redox processes. However the most readily available source of energy for organisms comes from the phosphoryl transfer chemistry of ATP **(Figure 1.16)**. Nature has come up with two ways to link redox reactions with ATP formation.

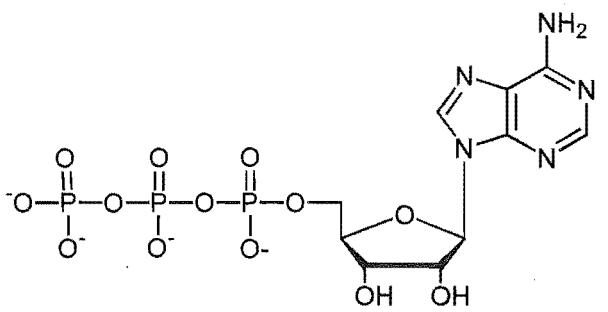

Figure 1.16. ATP, nature's energy currency.

In substrate-level phosphorylation, a favourable redox reaction is coupled to ATP formation by chemical intermediates. A large part of the energy released in the electron transfer reaction is retained in an energy rich bond such as a thioester. ATP

formation from ADP and inorganic phosphate can then take place in a reversible group transfer reaction. For example the oxidation of glyceraldehyde-3-phosphate to the carboxylic acid is thermodynamically favourable by ~50kJ/mol under standard conditions. This is more than enough to drive the synthesis of ATP from ADP and phosphate, which is endothermic by about 32kJ/mol. In order to couple the oxidation reaction with ATP formation, the carboxylic acid is trapped in an activated form as 1,3-bisphosphoglycerate, an acyl phosphate. Acyl phosphates have a high phosphate group transfer potential, having a free energy of hydrolysis of ~ -42kJ/mol. 1,3-Bisphosphoglycerate can transfer a phosphoryl group to ADP in a thermodynamically favourable reaction. The actual mechanism **(Figure 1.17)** involves a thioester intermediate as well.

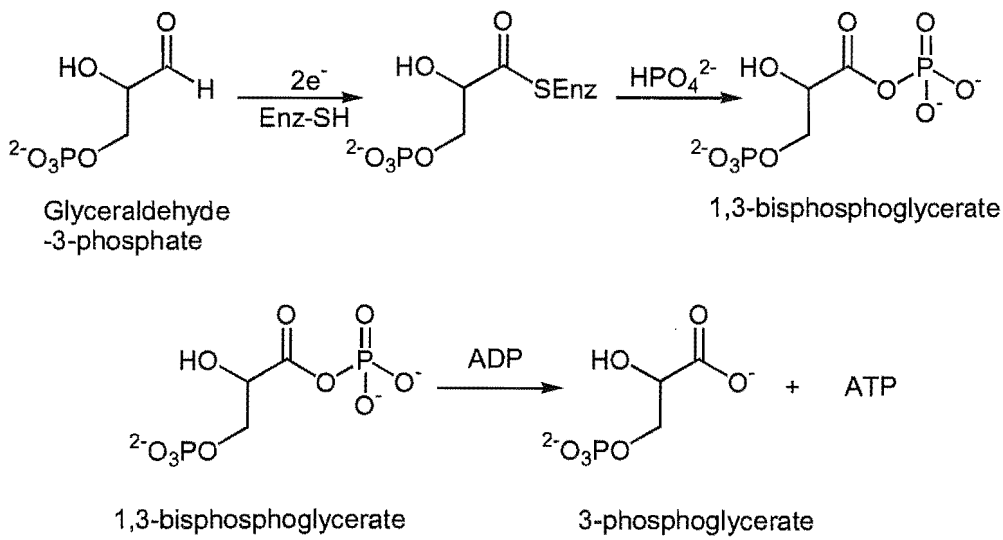

Figure 1.17. Substrate-level phosphorylation.

In carrier-level phosphorylation, ATP formation is coupled to electron transfer, either between two electron carriers, or between an electron carrier and a terminal electron acceptor or donor. The proteins involved in carrier-level phosphorylation are organised within a membrane and form a chain, with electrons flowing from one carrier to the next. Electron transfer is coupled to translocation of protons across the membrane, generating an electrochemical proton gradient between the two sides of

the membrane. The electrochemical potential of this gradient is harnessed to synthesise ATP, by coupling the favourable return of protons to ATP formation **(Figure 1.18)**.

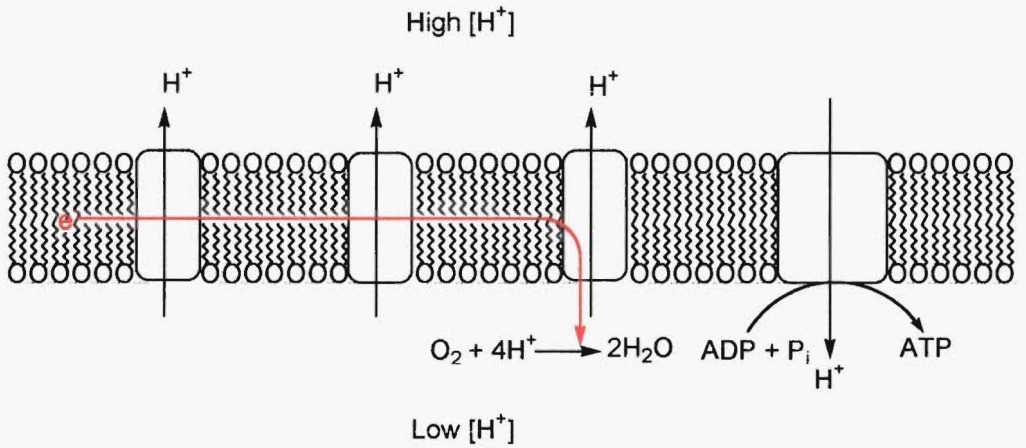

Figure 1.18. Carrier-level oxidation.

The complex nature of carrier-level phosphorylation, which requires many enzymes and a membrane, suggests an advanced level of evolution. It is most likely that substrate-level phosphorylation arose first, followed by carrier-level phosphorylation.

1.7.2 Coupling reactions.

Many biosynthetic reactions involve the joining of two small molecules with the release of a molecule of water. Such dehydrating condensation reactions are used to make all the biological polymers; proteins from amino acids, polysaccharides from sugars, and nucleic acids from nucleotides. These dehydration reactions are unfavourable in an aqueous environment and can only take place with a supply of energy. In almost all cases, the condensation reaction is coupled with the hydrolysis of ATP, either directly or indirectly.

The only way that chemical energy can be transferred from one chemical reaction to another is for the two reactions to have a common intermediate. For example, the

synthesis of glutamine involves the condensation of ammonia with the γ-carboxyl group of glutamate. The γ-carboxyl group is first activated as an acyl phosphate in the first step and glutamine is formed in the second step by the condensation of ammonia and γ-glutamyl phosphate **(Figure 1.19)**.

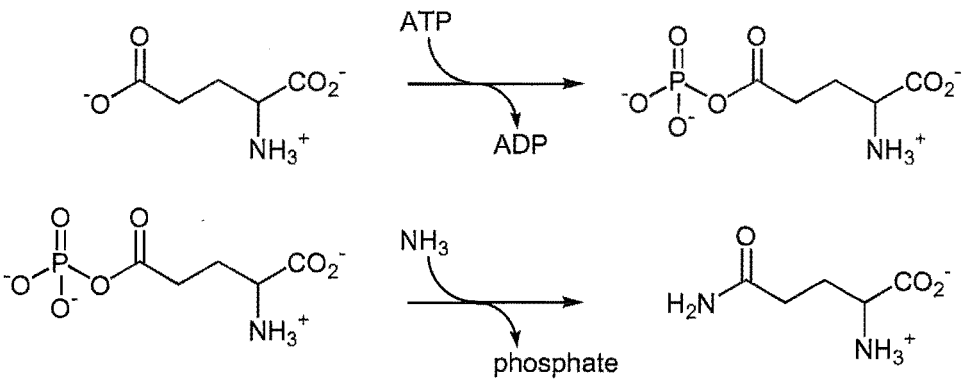

Figure 1.19. Glutamine synthesis, showing the common intermediate (an acyl phosphate) required to couple ATP hydrolysis with glutamine formation.

Because water is not a participant in these reactions, the free energy of ATP hydrolysis is not lost. Most of it is retained in the activated intermediate and can be used for the second step.

1.8 Why Ferrous Ions and Phosphates?

The objective of this thesis is to investigate the role of Fe(II) in phosphoryl transfer reactions in the hope of shedding light on the origins of phosphate metabolism. In extant organisms, phosphate metabolism is very rarely associated with iron chemistry. The few exceptions include acid phosphatases and ferritins. Acid phosphatase is an enzyme that catalyses the hydrolysis of phosphate esters and requires two metal ions at its active site; usually at least one of these is a Fe^{3+} ion. Ferritin is a protein that stores iron in the form of a mixed ferric hydroxide/ferric phosphate precipitate. In order to release iron, the Fe^{3+} must first be reduced to Fe^{2+}, presumably because Fe^{2+} is more labile. The lability of Fe^{2+} in the presence of

phosphate may be important in the chemistry addressed in the following chapters. Ferritin may also serve as a store of inorganic phosphate. The chemistry of iron and phosphate may have been more intimately related early in the history of the earth. At that time, iron was much more plentiful because it was largely present as Fe(II), whereas now the availability of iron is limited by the solubility of ferric hydroxide, which is extremely insoluble.

1.8.1 The role of iron in biochemistry.

Iron is essential for all life, and plays an important role in many reactions. It is one of the most versatile catalysts that nature has acquired, being involved in both electron transfer reactions and group transfer reactions. The type of iron centres found in proteins can be divided into a number of classes, comprising iron-sulfur clusters, Fe-O-Fe clusters, haem-iron, and other mononuclear iron enzymes.

Proteins containing iron-sulfur clusters **(Figure 1.20)** are most commonly involved in electron transfer reactions, such as the ferrodoxins and the iron-sulfur proteins involved in the electron transport chain of the mitochondria. The iron-sulfur centres involved in electron transfer are usually coordinatively saturated. However, a number of iron-sulfur centres have been found that bind substrates, such as aconitate and dihydrogen, allowing acid-base or free radical chemistry. These centres typically have one iron atom that is able to exchange with iron in solution, and it is this iron which binds substrate. Some iron-sulfur proteins are believed to be of very ancient origin,[34] lending support to the idea that FeS played a central role in the origin of life.

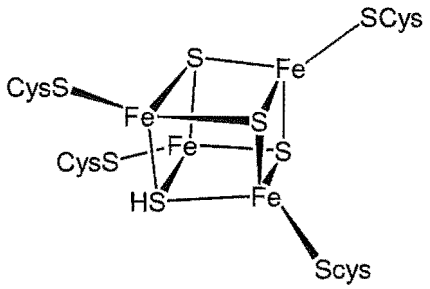

Figure 1.20. Basic structure of the Fe_4S_4 cluster.

The Fe-O-Fe cluster is found in haemerythrins **(Figure 1.21)**, oxygen transport proteins that bind oxygen as the peroxide dianion, O_2^{2-}. It is also found in two enzymes that are involved in radical chemistry, iron ribonucleotide reductase and methane monooxygenase. These two enzymes both generate radical centres by the abstraction of a hydrogen atom.

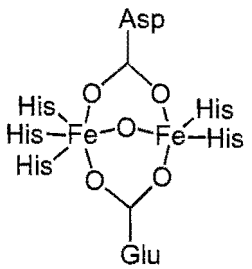

Figure 1.21. Active site of haemerythrin. Oxygen binds to the iron with five ligands.

Haem-iron proteins are usually associated with electron transport or the binding and activation of small molecules. The haem unit consists of a porphyrin ring that binds an iron atom at its centre. The porphyrin ring is roughly planar, and the four nitrogens occupy four of the coordination sites of the iron **(Figure 1.22)**. The proteins involved in electron transport contain an iron that is coordinatively saturated; those involved in binding substrates have one vacant site on the iron. Some haem-iron proteins bind small molecules reversibly; for example the oxygen transport proteins myoglobin and haemoglobin. Other haem-iron proteins are enzymes involved in redox chemistry, such as cytochrome oxidase and cytochrome

P_{450}. Cytochrome oxidase catalyses the four-electron reduction of dioxygen to water, while cytochrome P_{450} is involved in the hydroxylation of inert organic compounds.

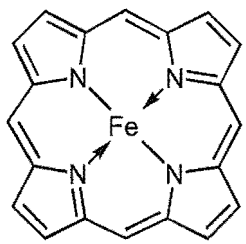

Figure 1.22. Basic structure of the haem-iron unit. A variety of organic substituents can occur on the outside of the porphyrin ring.

Finally, there is a range of enzymes that contain an iron atom bound to the protein via oxygen or nitrogen donors. The various enzymes usually involve the binding and activation of O_2. One of the few types of iron-containing enzymes not involved in redox chemistry are the purple acid phosphatases, which carry out the hydrolysis of a variety of phosphate esters.

The dependence of nature on iron is somewhat unfortunate, as iron is the limiting nutrient in many environments. This has meant that organisms have had to develop special mechanisms for the uptake and storage of iron. This unusual dependence on an element with low bioavailability is believed to be due changes in the amount of oxygen present during the history of the earth. Iron is present in natural waters at very low levels, because of the very low solubility of ferric hydroxide (K_{sp}~10^{-36}).[35] The low bioavailability of iron is due to the development of photosynthesis, which resulted in a gradual increase in the level of oxygen in the atmosphere. In the presence of oxygen, Fe(III) is the most stable state of iron, whereas under anaerobic conditions, such as during the first billion years of life, Fe(II) is more stable. Fe(II) is much more soluble in water than Fe(III), as it does not form insoluble hydroxides to the same extent. Thus, during the early history of life, iron, in the form of Fe(II), was readily available and its versatility as a catalyst was quickly exploited. By the time that oxygen levels began to rise, iron was an essential element for life, and this meant

that organisms had to adapt by either evolving ways of sequestering iron, or by restricting themselves to anaerobic environments where Fe(II) was available.

1.8.2 The role of phosphates in biochemistry.

Phosphate compounds play a number of important roles within the cell. Phosphoanhydrides are readily available sources of free energy. A phosphoanhydride, ATP, is the energy currency of all cells. Energy obtained from the environment, from sunlight or from organic material, is channelled into ATP synthesis. When the cell requires a source of energy, ATP is almost always involved, directly or indirectly. In water phosphoanhydrides are thermodynamically unstable with respect to hydrolysis. However they are kinetically stable at physiological pH, an important attribute if they are to be of use to a cell; hence they can be used to store energy.

The backbones of nucleic acids also contain phosphate. Phosphodiester linkages between sugars form the backbone of DNA and RNA **(Figure 1.23)**. Like phosphoanhydrides, phosphodiesters are thermodynamically unstable but kinetically stable. This allows the nucleic acids to be easily manipulated provided that catalysts are present. In the absence of catalysts, phosphodiesters are relatively stable, a useful prerequisite for a store of genetic information.

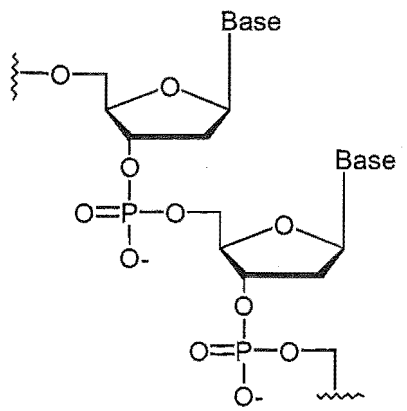

Figure 1.23. Phosphodiester backbone of DNA.

Biological membranes also make use of phosphate. Membranes are composed of molecules containing hydrophobic and hydrophilic sections that form lipid bilayers. In water, the hydrophobic segments aggregate together and exclude water, with the hydrophilic portions at the surface interacting with the water. The most common lipids in biological membranes are phosphoglycerides, which are esters of glycerol-3-phosphate **(Figure 1.24)**.

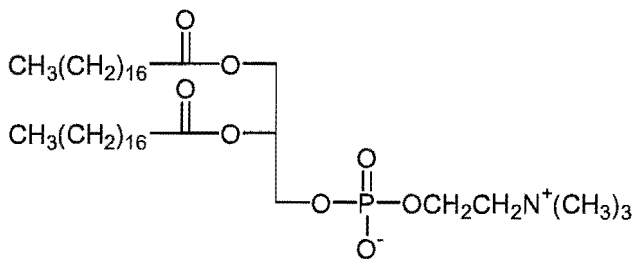

Figure 1.24. Phosphatidylcholine, an example of a phosphoglyceride.

Inorganic polyphosphate, a polymer of up to several hundred phosphates linked by phosphoanhydride bonds, is also found in every cell. Inorganic polyphosphate is believed to have a number of functions, including: a store of inorganic phosphate; chelation of metal ions; buffering against alkali; and also as a substitute for ATP as a phosphoryl donor.[36]

The widespread use of phosphate leads to two questions; why are phosphates so useful, and how did phosphates become to be so important? The first question can be answered on the basis of the chemical properties of phosphates. This was succinctly addressed by Frank Westheimer in an article entitled "Why Nature Chose Phosphates".[37] Westheimer argued that phosphate is suited for all of its roles because it is always anionic at physiological pH. The pK_a's of phosphoric acid are 1, 6.5 and 13, which means that at neutral pH it carries a negative charge of at least 1. Polyphosphates and phosphate monoesters and diesters are likewise always negatively charged at neutral pH. The kinetic stability of phosphate compounds is largely due to this anionic nature. Nucleophilic attack at phosphorus is inhibited due

to electrostatic repulsion between the nucleophile and the negative charge. The negative charge that phosphate always carries is also essential for it to be useful as the polar component of lipid molecules. Many small metabolites also contain phosphate and Westheimer has argued that one of the reasons for this is to ensure that the molecules are retained within the cell, since ionic compounds are unable to pass through the hydrophobic lipid membrane.

While it can be argued that phosphate is uniquely suited for its roles, it does not explain how phosphate became such a dominant part of biochemistry. The prevalence of phosphate in biology implies that a primitive organism incorporated phosphates early on. This poses a major problem in developing a model for the origin of life, since the concentration of phosphate in seawater is very low, about 10^{-8} mol/L, and it is unlikely to have been significantly higher on the early earth. The scarcity of soluble phosphate is due to the insolubility of phosphate in the presence of divalent or trivalent metal ions. Polyphosphates, such as pyrophosphate, are somewhat more soluble, however they are very rare minerals and are unlikely to have been a significant source of phosphate. Pyrophosphate, and other polyphosphates, has been implicated as an evolutionary precursor to ATP.[38] This is supported by the discovery of a number of reactions that utilise pyrophosphate as a phosphoryl donor, rather than ATP. If pyrophosphate was to be an early source of energy and of phosphate then any emerging metabolism would have to have found a way of synthesising and utilising pyrophosphate. Pyrophosphate and other polyphosphates are unlikely to have been present in great quantities on the early earth, although a small amount of a pyrophosphate-containing mineral has been found,[39] and evidence has also been found for the production of pyrophosphate and higher polyphosphates in volcanic eruptions.[40] Whether or not this represented a significant source of polyphosphates is unclear.

To be able to incorporate phosphate into a metabolism requires that a source of phosphate be found. As explained above, the source is unlikely to be soluble inorganic phosphate or polyphosphates. This leaves us with insoluble sources of

phosphate. A metabolism based on surface catalysis would have a distinct advantage over a solution-based metabolism when it came to incorporating phosphate. The mineral surface may contain phosphate that could potentially react with organic compounds bound to the surface. Phosphate has been found to be a minor component of many minerals, including FeS.[41] The phosphorylated compound could then undergo further reaction and still be retained by the surface, since the phosphate is anionic. This would mimic the role of phosphate in retaining small molecules within a membrane; instead phosphate would retain small molecules on the mineral surface.

1.8.3 Pyrophosphate as a precursor of ATP.

An emerging metabolism would require a source of readily available energy in order to drive thermodynamically unfavourable reactions, fulfilling the role that ATP plays in modern cells. ATP is regarded as too complex to have been the first phosphoanhydride used as an energy source. ATP formation requires the condensation of adenine, ribose and tripolyphosphate, an unlikely event without a prior source of dehydrating power. As we have already seen, both adenine and ribose would have been present on the early earth so the eventual emergence of ATP is not inconceivable. For this reason inorganic polyphosphates have been put forward as an evolutionary precursor to ATP. This notion receives some support from contemporary biochemistry. Polyphosphates can act as a phosphoryl donor for a number of enzymatic reactions in some organisms. Pyrophosphate metabolism is closely linked to acetyl phosphate metabolism in some thermophiles,[42] a set of reactions that utilise the interconversion of thioesters with polyphosphates (see figure 1.25). In some photosynthetic bacteria, pyrophosphate can also be formed using light energy, in a manner analogous to the photophosphorylation of ATP.[43]

Wächtershäuser[26] and de Duve[27] have argued that phosphate was incorporated into an emerging metabolism with the help of thioesters. Thioesters are high-energy acyl compounds, with a free energy of hydrolysis very similar to the free energy of hydrolysis of a phosphoanhydride bond, about -31kJ/mol. Polyphosphates and

thioesters can be interconverted through the intermediacy of acyl phosphates (**Figure 1.25**). This interconversion is still very important in biochemistry, leading to the synthesis of ATP and GTP during glycolysis and the citric acid cycle, as well as being the method of activating amino acids prior to protein synthesis.

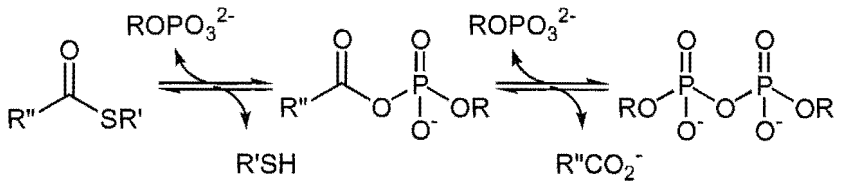

Figure 1.25. Thioester-polyphosphate interconversion.

This thesis concerns the incorporation of phosphate into an emerging metabolism, and can be divided into two sections. The first section encompasses chapters two and three and looks at ways of forming phosphoanhydride bonds in a manner analogous to the formation of ATP in substrate-level phosphorylation. Chapter two uses the simplest acyl phosphate, acetyl phosphate, as a phosphoryl donor, while in chapter three, phosphoenolpyruvate is used as a phosphoryl donor. The second section, chapter four, addresses the chemistry of polyphosphates in an effort to understand how polyphosphates could be utilised by an emerging metabolism.

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2 Acetyl Phosphate Chemistry.

2.1 Introduction.

2.1.1 Acyl phosphates in biology.

Acyl phosphates are high-energy compounds that are important in biochemistry. As discussed in chapter 1, they are formed as intermediates in number of energy coupling reactions, such as the formation of polyphosphates from thioesters and the activation of carboxylic acids. Their usefulness is a result of their ability to react with nucleophiles at either of two electrophilic sites: at the carbonyl carbon; or at phosphorus **(Figure 2.1)**.

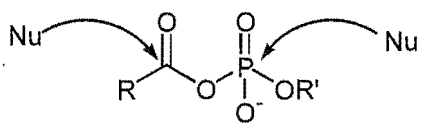

Figure 2.1. Electrophilic centres of acyl phosphates.

Nucleophilic attack at the carbonyl carbon leads to acyl transfer; for example, in the formation of aminoacyl t-RNA, an important part of protein synthesis **(Figure 2.2)**.

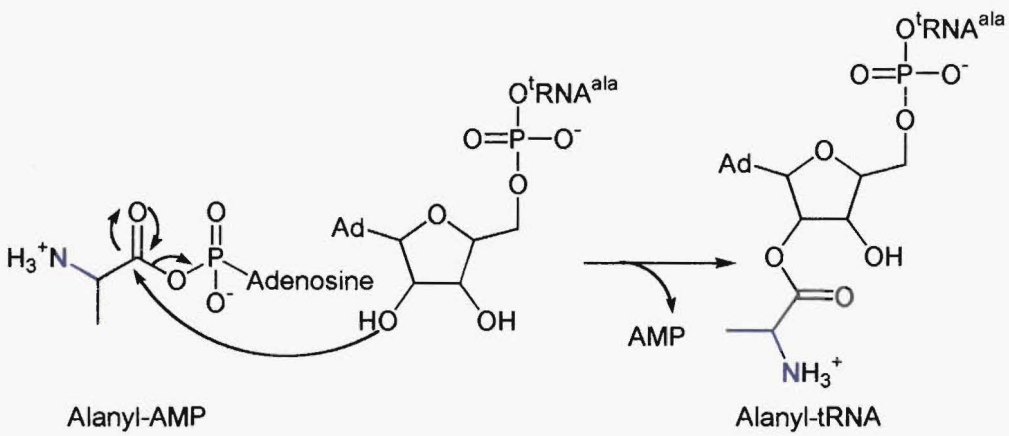

Figure 2.2. Acyl transfer from an acyl phosphate.

Attack at phosphorus results in phosphoryl transfer; an example of this is the formation of ATP from 1,3-bisphosphoglycerate during glycolysis **(Figure 2.3)**.

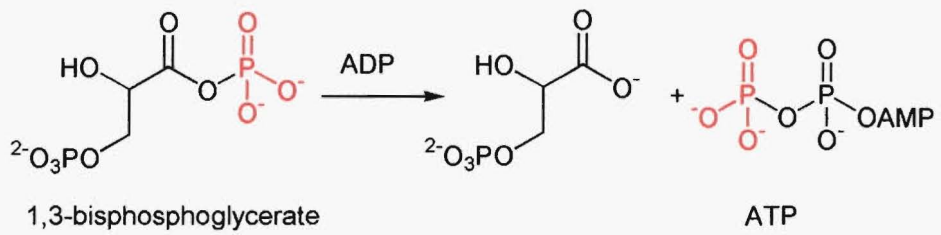

Figure 2.3. Phosphoryl transfer from an acyl phosphate.

The ambident nature of acyl phosphates begs the following question. What determines whether a nucleophile reacts at the carbon or at phosphorus? The reactivity of acyl phosphates has been studied since their biological importance was first realised in the 1940's.[1] Numerous studies have shown that under most circumstances nucleophilic attack occurs at carbon, resulting in acyl transfer.[2] Phosphoryl transfer is generally less favourable, partly because of electrostatic repulsion between the nucleophile and the phosphoryl group; if this can be reduced then phosphoryl transfer may take place. Biological systems achieve this in two ways, through the use of metal ions or amines. Metals ions reduce the electron density surrounding the phosphoryl group by binding to it. Amines act as

nucleophilic catalysts, as they are effective uncharged nucleophiles and are able to form reactive *N*-phosphoryl intermediates. These can subsequently undergo a second phosphoryl transfer reaction.

The formation of phosphoanhydride bonds from acyl phosphates and phosphates is one of the most important phosphoryl transfer reactions that acyl phosphates undergo. It is also relatively easily studied since the product, a polyphosphate, is comparatively stable and can be quantified. Studying phosphoryl transfer to nucleophiles other than phosphate is hindered by the instability of many phosphorylated species. This is particularly true in the case of amines, as the *N*-phosphorylated intermediate is unstable with respect to hydrolysis. Trapping the *N*-phosphorylated intermediate with a second phosphate to form pyrophosphate is one of the few unequivocal ways of demonstrating that phosphoryl transfer has occurred.

2.1.2 Metal ions as catalysts for phosphoryl transfer.

Metal ions are able to coordinate with acyl phosphates and withdraw electron density from the phosphate group **(Figure 2.4)**. This may encourage nucleophilic attack at phosphorus by reducing electrostatic repulsion between the nucleophile and the phosphate group.

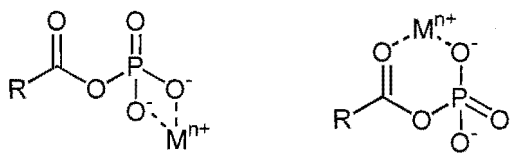

Figure 2.4. Possible coordination modes of metal ions with acyl phosphates.

Vieyra and co-workers[3] have shown that Mg^{2+}, Ca^{2+} and Sr^{2+} are able to catalyse the formation of pyrophosphate from acetyl phosphate and inorganic phosphate in yields of up to 8%. High concentrations of sodium ions (~7M) were also shown by Herschlag and Jencks[4] to catalyse pyrophosphate formation in yields of up to 10%.

Meade[5] found that Mg^{2+} promotes pyrophosphate formation, although in low yield (<2%), but was unable to repeat the work of Herschlag and Jencks. Meade[5] investigated the ability of Fe^{2+} to catalyse phosphoryl transfer and found that pyrophosphate was formed in yields of up to 15%. Other metal ions may also be effective catalysts for phosphoryl transfer.

Many metal ions are able to catalyse the hydrolysis of acyl phosphates.[6,7] This is particularly true of divalent and trivalent metal ions that coordinate strongly with phosphate groups. Fife and Pujari[8] showed that Cu^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+} increased the rate of hydrolysis of 1,10-phenanthroline-2-carbonyl phosphate, an acyl phosphate that is able to bind metal ions very strongly. Ni^{2+}, Co^{2+} and Zn^{2+} all increased the rate of hydrolysis by a factor of $\sim 10^7$, while Cu^{2+} increased the rate of hydrolysis by a factor of $\sim 10^{10}$. These large increases in rate are due, in part, to the tight binding of the metal ion in close proximity to the acyl phosphate. However, metal ions have only a small effect on the hydrolysis of acetyl phosphate since it does not bind metal ions very tightly. Divalent metal ions generally catalyse the hydrolysis of the acetyl phosphate dianion but not the monoanion, presumably because the dianion is able to coordinate the metal ion more strongly. Monovalent metal ions generally do not affect the reactions of acyl phosphates, unless they are present in very high concentrations.

2.1.3 Fe(II) as a catalyst for phosphoryl transfer.

This chapter describes an investigation of the ability of ferrous ions to act as a catalyst for phosphoryl transfer, furthering the work of Meade.[5] The aim is to understand how different factors, such as the pH and the amount of Fe^{2+}, affect phosphoryl transfer. Ferrous ions were chosen for a number of reasons. Fe(II) has the potential to be an excellent catalyst as it forms labile complexes with a wide variety of nitrogen, oxygen and sulfur ligands. As mentioned in the previous chapter, Fe(II) was also plentiful at the origin of life and is now an essential element for all life. Hence the ubiquitous phosphoryl transfer chemistry of metabolism developed in the

presence of Fe(II). Phosphate is known to occur as a minor component of many minerals, including iron sulfide.[9] Phosphate forms insoluble precipitates with many di- and tri- valent metal ions, including Fe(II), which contributes to the low bioavailability of phosphates in solution. This ability to form precipitates may be advantageous in catalysis as it provides a mechanism for concentrating reactants onto the surface of the precipitate. Other precipitates, such as montmorillonite and apatite, have been shown to be effective catalysts for a number of "prebiotic" reactions.[10,11]

2.1.4 Catalysis of phosphoryl transfer by precipitates.

As mentioned above, phosphate forms precipitates in the presence of many divalent metal ions. Other phosphate compounds, such as acetyl phosphate and pyrophosphate also form precipitates in the presence of these metal ions. Preliminary results indicated that in the reactions described here, pyrophosphate formation took place on or within the ferrous phosphate precipitate. As any changes in the precipitate are likely to affect pyrophosphate formation, it is worthwhile discussing briefly the nature of the precipitates that may form, and how this may affect catalysis.

For any metal ion, the solubility product with phosphate is likely to be smaller than the solubility product with acetyl phosphate, so in the presence of a limited amount of metal ion selective precipitation of phosphate will occur. At low levels of metal ion, the precipitate will consist mainly of the metal ion and phosphate, while at higher levels of metal ion, when most of the phosphate is bound within the precipitate, more acetyl phosphate will be incorporated into the precipitate. The presence of acetyl phosphate may alter the structure of the precipitate, as the acetyl group will disrupt the metal-phosphate lattice. This may make the precipitate more dynamic, increasing the rate of exchange of ligands in the precipitate. The ability of acetyl phosphate to alter the nature of the precipitate is highlighted by the work of Meyer-Fernandes and Vieyra.[12] They found that pyrophosphate formation from acetyl phosphate did not occur in the presence of a calcium phosphate precipitate, but

in the presence of a precipitate containing a large amount of acetyl phosphate, pyrophosphate formation did take place.

The ferrous phosphate precipitate may promote pyrophosphate formation in a number of ways. The unfavourable electrostatic interactions between the phosphate and the acetyl phosphate may be reduced when the substrates are bound to Fe(II). The Lewis acid nature of Fe^{2+} may enhance the electrophilicity of the phosphorus centre by polarising the phosphorus-oxygen bonds. The precipitate may enhance phosphoryl transfer by concentrating the reactants, and possibly through positioning the reactants in the proper orientation for reaction **(Figure 2.5)**. Sequestering the acetyl phosphate within the precipitate, away from the solvent, may also lower the rate of hydrolysis of acetyl phosphate.

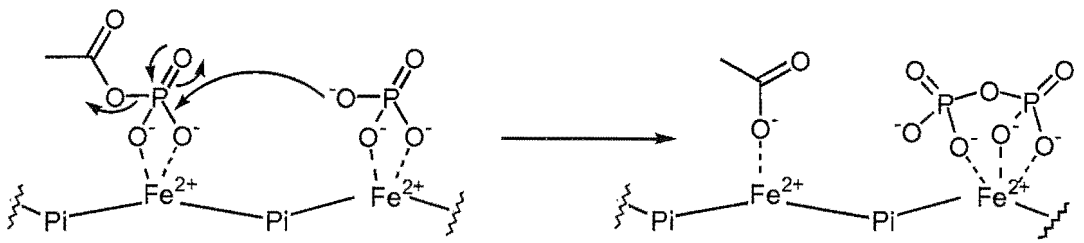

Figure 2.5. Possible mechanism for pyrophosphate formation.

2.2 Technical Aspects.

2.2.1 Analysis of phosphate species.

All phosphoryl transfer reactions in this thesis were analysed by ^{31}P NMR. This technique is eminently suitable for the study of phosphoryl transfer reactions, as it is able to provide the relative concentrations of all phosphate species in solution. It is suitable for any phosphoryl transfer reaction provided the phosphate species are soluble. However, NMR is not particularly suitable for analysing very dilute

solutions since it is not very sensitive. Hence small amounts of phosphoryl transfer (<1%) are unlikely to be detected.

Alternative techniques typically involve the determination of total phosphate before and after acid hydrolysis, or the use of radiolabelled phosphates and chromatography. These were deemed unsuitable, as they do not give complete information on the phosphate species present in a solution. Additionally, working with ^{32}P requires specialist equipment to deal with radioactive material.

2.2.2 Problems associated with the use of Fe(II).

The use of Fe(II) presents a number of experimental difficulties with respect to analysing phosphoryl transfer reactions. These are the sensitivity of Fe(II) to oxidation, the insoluble nature of ferrous phosphates, and the paramagnetic nature of high spin Fe(II). All reactions involving Fe(II) were performed under an atmosphere of argon using syringe techniques to prevent oxidation. While this limits the amount of oxidation that occurs, there is no guarantee that there was no Fe^{3+} present. The presence of Fe^{3+} will be addressed in a later section. Insoluble ferrous phosphates are not suitable for analysis by NMR, so a method of desorbing the phosphates from the metal ion was required. Cyanide was used to solubilise the phosphate species. Cyanide binds very tightly to Fe(II) to form the substitutionally inert ferrocyanide complex, $Fe(CN)_6^{4-}$. Complexing the Fe(II) with cyanide also prevents any paramagnetic effects being observed in the NMR, as the ferrocyanide contains low spin, diamagnetic Fe(II). Paramagnetic ions can lead to very broad signals in a NMR spectrum if the paramagnetic centre interacts with the nucleus being studied.

2.2.3 Fe(II) or Fe(III)?

There is always the possibility when dealing with Fe(II) that oxidation to Fe(III) occurs. The standard reduction potential, $E^{\varnothing}$, for the reduction of Fe^{3+} in aqueous

solution is 0.77V; another way of saying this is that the reverse reaction, the oxidation of Fe^{2+} has a potential of –0.77V. Since a positive value of $E^{\varnothing}$ signifies a thermodynamically favourable reaction, this suggests that Fe^{2+} is stable in solution. This is the case, provided oxygen is not present. In the presence of oxygen, the oxidation of Fe^{2+} is thermodynamically favourable, having a standard reduction potential of 0.44V.

$$4Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O \quad E^{\circ} = 0.44V$$

The reduction potential is pH sensitive, and at neutral pH it is 0.04V, still thermodynamically favourable. However, a reduction potential of 0.04V does not provide the large overpotential that is often required for fast reaction and as a consequence oxidation of Fe^{2+} is generally slow in the absence of catalysts.

As stated above, all reactions were carried out under an atmosphere of argon. Reactions that lasted less than one day also had a positive pressure of argon applied to prevent back diffusion of air. Since this would have required large amounts of argon for longer reaction times, experiments that lasted for more than a day were prepared under an atmosphere of argon and then incubated in argon-filled chambers.

To determine the amount of oxidation that occurs during a typical run, a control experiment to test the degree of oxidation was assayed for Fe^{2+} and Fe^{3+} after the reaction was complete. The assay was based on the absorption of 1,10-phenanthroline complexes of iron.[13] This method enables the concentration of Fe(II), and the total Fe(II) and Fe(III) concentration to be measured. At a wavelength of 512nm, only the Fe(II) complex absorbs significantly, while both Fe(II) and Fe(III) complexes absorb equally at 396nm.

The control reaction, which originally contained 0.075M Fe(II), was assayed after 1 hour at 50°C using this method. The concentration of Fe(II) was 0.076M, while the

total concentration of iron (Fe(II) and Fe(III)) was 0.078M, representing a 3% level of oxidation. The difference between the starting concentration of iron and the final concentration of iron is probably due to a combination of errors in measuring the original solution, and the evaporation of water over the course of the reaction.

Although a number of precautions were taken to prevent oxidation from occurring, the possibility that phosphoryl transfer is catalysed by trace amounts of Fe^{3+} can not be completely ruled out. Pyrophosphate formation was not observed in reactions where Fe^{3+} was the only source of iron, however analyses of these reactions were complicated by the inability to remove all the phosphates from the Fe^{3+}. While the presence of Fe^{3+} complicates any detailed analysis of the reaction, it does not alter the fact that phosphoryl transfer does occur in the presence of Fe(II). Even on the early earth, which had a mildly reducing environment, there would always have been small amounts of Fe^{3+} present. For the rest of this thesis, the discussion will be centred on Fe^{2+} and it will be assumed that trace amounts of Fe^{3+} do not interfere with the phosphoryl transfer results obtained.

2.2.4 Synthesis of dilithium acetyl phosphate.

A source of pure acetyl phosphate was required before any phosphoryl transfer reactions could be investigated. Commercial dilithium acetyl phosphate, from Aldrich, was found to contain small amounts of phosphate and pyrophosphate. Obviously the presence of pyrophosphate is undesirable, as it is the product of the reaction being studied. There are a number of syntheses of acetyl phosphate in the literature; of these the method of Avison[14] was the most suitable as it provided dilithium acetyl phosphate as a solid. Acetic anhydride was used to acetylate inorganic phosphate in an aqueous pyridine solution **(Figure 2.6)**. Acetyl phosphate was obtained as the dilithium salt by adding a solution of lithium hydroxide and then precipitating the product from a dilute alcoholic solution. Dilithium acetyl phosphate was obtained in a yield of 66% after recrystallisation and was free from contaminants as judged by ^{1}H and ^{31}P NMR.

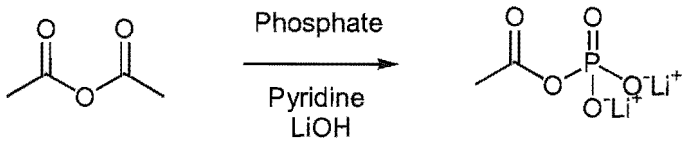

Figure 2.6 Synthesis of acetyl phosphate.

2.3 Phosphoryl Transfer Reactions of Acetyl Phosphate Catalysed by Fe(II).

The ability of Fe(II) to catalyse phosphoryl transfer reactions of acetyl phosphate is likely to be affected by a number of environmental factors, such as the pH of the solution and the amount of Fe(II) present. The influence of these factors on phosphoryl transfer to water or to inorganic phosphate may be complex. For instance, the reactivity of acetyl phosphate may be altered, or the nucleophilicity of inorganic phosphate may be changed. By using ^{31}P NMR to analyse the reactions, two rates can be monitored; the disappearance of acetyl phosphate and the formation of pyrophosphate. The rate of acetyl phosphate loss provides information about the reactivity of acetyl phosphate towards nucleophiles under the conditions used. This will include nucleophilic attack at carbon as well as at phosphorus. The amount of pyrophosphate formed during the reaction furnishes information about the partitioning of acetyl phosphate between hydrolysis and phosphorolysis.

2.3.1 The effect of pH on phosphoryl transfer.

The pH of the reaction is likely to have a significant effect on the reactivity of acetyl phosphate and the formation of pyrophosphate since acetyl phosphate, inorganic phosphate and pyrophosphate all have a number of dissociable protons. The degree of protonation of the phosphate groups will also effect the strength of binding to any metal centres. Fe(II) also displays pH-dependent chemistry in aqueous solution, notably the formation of insoluble ferrous hydroxides in basic solution, as well as insoluble ferric hydroxides after oxidation.

The hydrolysis of acetyl phosphate was shown by Koshland[15] to be pH-independent over the pH range 3-9. However the phosphorolysis of acetyl phosphate in the presence of sodium or calcium ions is pH-dependent. Herschlag and Jencks[4] reported that the yield of pyrophosphate from acetyl phosphate and inorganic phosphate in the presence of 7.3M sodium perchlorate increased as the pH was increased from 3 to 7.5. The yield of pyrophosphate from the reaction at pH 7.5, when phosphate and acetyl phosphate are predominately dianions, was 11.7%. The yield dropped to 4.2% at pH 3, when the reacting species are the monoanions of acetyl phosphate and phosphate.

Vieyra and coworkers[3] investigated the formation of pyrophosphate from acetyl phosphate and inorganic phosphate in the presence of Ca^{2+} over the pH range 5 – 9, and found that the maximum yield of pyrophosphate occurred at pH 7. The increase in the rate of pyrophosphate formation with increasing pH from 5 to 7 suggests that the dianions of acetyl phosphate and phosphate are important. Pyrophosphate formation decreased above pH 7, coinciding with the appearance of a calcium phosphate precipitate; this may inhibit pyrophosphate formation in solution by sequestering both calcium and phosphate within the precipitate.

The effect of pH on the Fe(II) catalysed formation of pyrophosphate was studied over the range 5.5 – 7.5, a pH range typical of biological systems. For this study the initial concentrations of Fe^{2+}, acetyl phosphate and phosphate, were 0.05M. The pH was maintained using the buffers MES, PIPES or HEPES, depending on the pH required. These buffers were used since they do not interact significantly with metal ions.[16]

The pH-dependence of the rate of reaction of acetyl phosphate in the presence of Fe^{2+}, a function of both hydrolysis and phosphorolysis, is shown below (**Figure 2.7**). Also shown is the pH-dependence of pyrophosphate formation in the presence of Fe^{2+} (**Figure 2.8**).

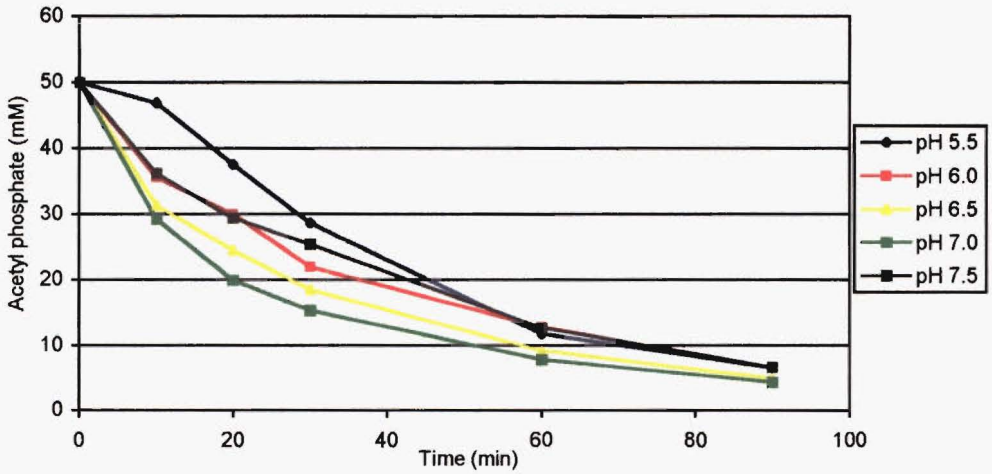

Figure 2.7. Effect of pH on the loss of acetyl phosphate.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4 and 0.05M $FeSO_4$ were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

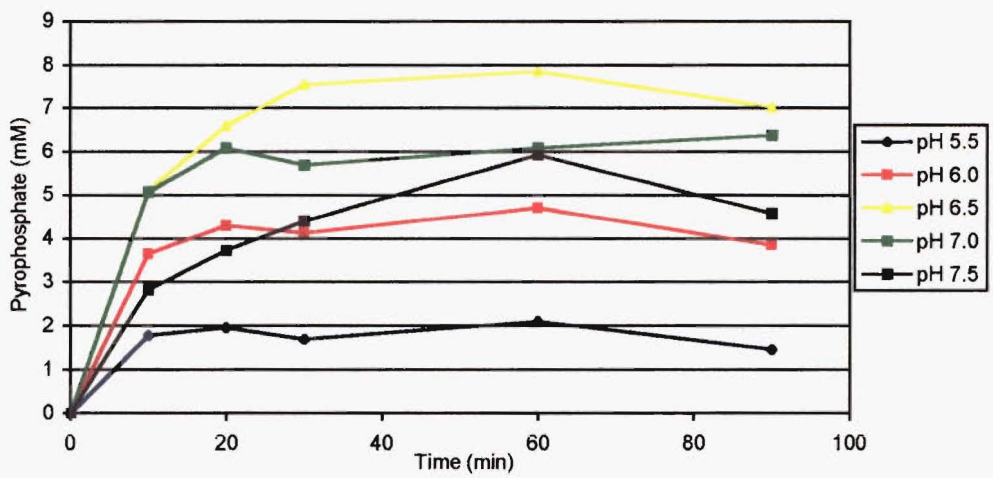

Figure 2.8. Effect of pH on the formation of pyrophosphate.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4 and 0.05M $FeSO_4$ were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

Under these conditions the rate of acetyl phosphate loss was fastest at a pH of 6.5-7. The formation of pyrophosphate essentially parallels the rate of acetyl phosphate

loss, with the yield of pyrophosphate reaching a maximum of 15% at pH 6.5. This is to be expected, as the disappearance of acetyl phosphate is a combination of losses due to both hydrolysis and phosphorolysis. Since acetyl phosphate undergoes pH-independent hydrolysis over this pH range,[15] any increase in the rate of disappearance of acetyl phosphate can be attributed to an increase in the rate of phosphorolysis.

A possible explanation for the observed pH behaviour may be that pyrophosphate formation is dependent on the amount of phosphate species bound to Fe^{2+} in the precipitate. Higher pH increases the degree of ionisation of the phosphates and increases the amount of precipitate formed in the reaction. Indeed at a pH < 2, no precipitate is observed, as all of the phosphates are fully protonated. However in alkaline solutions Fe^{2+} forms insoluble polymeric ferrous hydroxides and this has the effect of reducing the amount of Fe^{2+} available to bind phosphates. The oxidation of Fe^{2+} to Fe^{3+} is also accelerated at high pH. These factors may be a reason why the formation of pyrophosphate is greatest at a pH just below 7.

These results are consistent with the work reported by Herschlag and Jencks[4], and by Vieyra and co-workers.[3] They also found that pyrophosphate formation increased with pH, and this suggests that the strength of binding of the phosphates to the metal ions is important. The dianions of phosphate and acetyl phosphate will coordinate with metal ions more tightly than the monoanions acetyl phosphate and phosphate will. The metal ions appear to promote pyrophosphate formation in two ways: by reducing the electrostatic interactions between the reactants; and by bringing the reactants together.

In the work of Vieyra and co-workers,[3] when Ca^{2+} was present, pyrophosphate formation was not associated with the precipitate that formed above pH 7. This is in contrast to the work described here; in the presence of Fe^{2+}, the formation of pyrophosphate is associated with the precipitate. Clearly the nature of the precipitate

is important in pyrophosphate formation. How the precipitate may change when the amount of Fe^{2+} available is increased is described in the next section.

2.3.2 How the amount of Fe(II) present affects phosphoryl transfer.

Another important factor affecting the rate of pyrophosphate formation is the amount of Fe^{2+} present. The relative ratio of phosphates to Fe^{2+} will affect both the amount of precipitation and the nature of the precipitate.

In solution, increasing the amount of metal ions has previously been shown to have a positive effect on pyrophosphate formation. Herschlag and Jencks[4] found that increasing the concentration of sodium ions promoted pyrophosphate formation. Their results indicated that up to seven sodium ions were involved in the reaction complex leading to the formation of pyrophosphate.

Vieyra and co-workers[3] observed that, in solution, pyrophosphate formation increased with increasing concentrations of Ca^{2+} or Sr^{2+}. A plot of the rate of pyrophosphate formation against the concentration of metal ions gave a sigmoidal curve, suggesting that at least two metal ions were required to bind to the reactants for pyrophosphate formation to occur. These results indicate that multiple metal ions are required in order to bring the reactants together, and to reduce the electrostatic repulsion between the phosphate groups.

To study the effect of varying the amount of Fe^{2+}, the initial concentration of Fe^{2+} was varied over the range 0 - 0.1M in the presence of 0.05M acetyl phosphate and 0.05M phosphate at a pH of 6.5. A pH of 6.5 was chosen as this had previously been shown to give the highest yield of pyrophosphate.

The effect of increasing the relative amount of Fe^{2+} on the rate of acetyl phosphate loss **(Figure 2.9)** and on the rate of pyrophosphate formation is shown **(Figure 2.10)**.

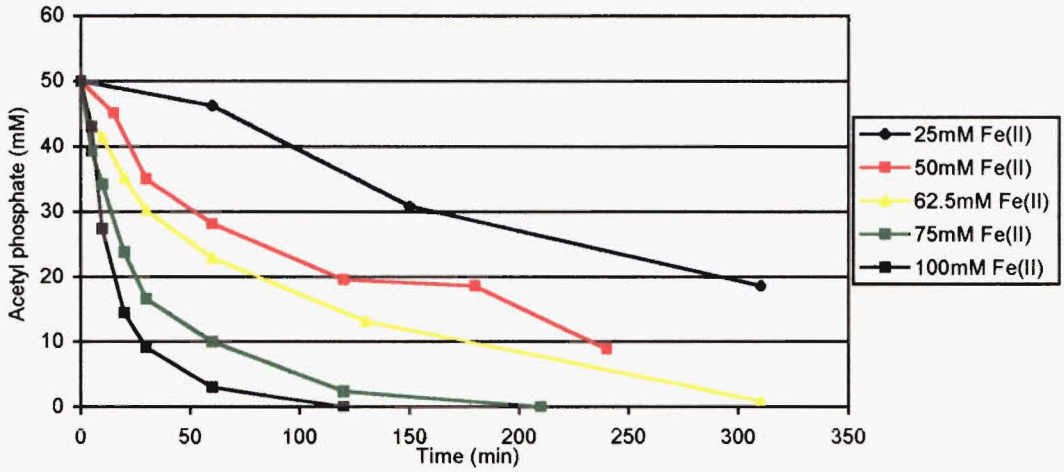

Figure 2.9. Effect of [Fe^{2+}] on the loss of acetyl phosphate.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4 and $FeSO_4$ were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

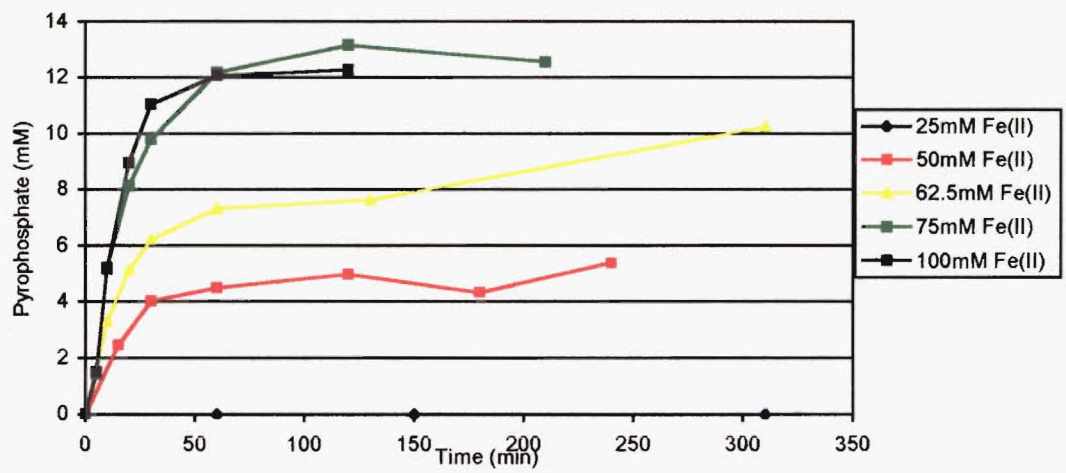

Figure 2.10. Effect of [Fe^{2+}] on the formation of pyrophosphate.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4 and $FeSO_4$ were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

The rate of acetyl phosphate loss increases as the amount of Fe^{2+} is increased. Pyrophosphate formation also increased with increasing amounts of Fe^{2+}, until a

maximum yield of ~25% was obtained at 0.075M Fe^{2+}. No pyrophosphate was observed when the initial concentration of Fe^{2+} was less than 0.05M.

The effect of increasing the amount of Fe^{2+} appears to be two-fold. The reactivity of acetyl phosphate increased, and the relative rate of phosphorolysis compared to hydrolysis is increased. The increased reactivity of acetyl phosphate is due to an increase in the rates of both hydrolysis and phosphorolysis. The increase in rate of hydrolysis can be most clearly seen by comparing the rates of pyrophosphate formation and of acetyl phosphate loss when the initial concentrations of Fe^{2+} were 75mM and 100mM Fe^{2+}. The rates of pyrophosphate formation are very similar, but the rate of acetyl phosphate loss is greater when 100mM Fe^{2+} was present compared to when 75mM Fe^{2+} was present. The increase in the rate of acetyl phosphate loss must then be due to a higher rate of hydrolysis. This is possibly due to the Lewis acidity of Fe^{2+}. The formation of a complex between Fe^{2+} and acetyl phosphate will reduce the electron density at the phosphoryl group and polarise both the carbonyl carbon and the phosphorus, making them both more susceptible to hydrolysis.

The rate of phosphorolysis increases up to a maximum level, as can be seen by the yields of pyrophosphate at the different Fe^{2+} concentrations. This is most likely due to changes in the precipitate as a result of an increase in Fe^{2+}, since increasing the amount of Fe^{2+} will result in more acetyl phosphate being present in the precipitate. A reason for the rate of pyrophosphate formation reaching a plateau above an initial concentration of 0.075M Fe^{2+} may be because all the phosphate species are bound within the precipitate at this concentration. If this were the case, then increasing the concentration of Fe^{2+} could no longer increase the amount of precipitated phosphate species.

The co-precipitation of phosphate and acetyl phosphate in the presence of Fe^{2+} will result in phosphate and acetyl phosphate interacting with multiple Fe^{2+} ions. The type of reactive complex that leads to the formation of pyrophosphate in the precipitate

may be analogous to the reactive complexes formed in solution in the presence of Ca^{2+} or Na^{+}, which also require multiple metal ions.

However, precipitates involving Ca^{2+} or Mg^{2+} are far less efficient at catalysing pyrophosphate formation than precipitates involving Fe^{2+} ions. This suggests that the nature of the mixed ferrous phosphate precipitate is different from precipitates of calcium or magnesium phosphates. The exact nature of the precipitates that are involved is unknown so very little can be said about why Fe^{2+} is a better catalyst than other divalent metal ions.

Thermodynamic and kinetic factors may influence the ability of a metal ion to catalyse pyrophosphate formation within a precipitate. An important thermodynamic factor may be the strength of binding of the various phosphates to the metal, as this will determine, in part, the ratio of bound phosphate to free phosphate. Interestingly the stability constant for the 1:1 complex with phosphate is higher for Fe^{2+} (log K ~ 2.6) than for Ca^{2+} (log K=1.33) or Mg^{2+} (log K=1.60).[17] The value for Fe^{2+} is only approximate, as the stability constants for transition metal phosphate complexes are notoriously hard to determine due to the insolubility of many of the complexes. A kinetic factor that may be relevant is the relative lability of the phosphate species bound to the metal centre, as this may effect the rate of reaction. Obviously for catalysis to occur, any product formed must dissociate from the metal centre before another reaction cycle can begin. The exchange rates of ligands bound to Ca^{2+} are on the order of 10^{9} s^{-1}, while for Fe^{2+} the rates are about 10^{6} s^{-1} and for Mg^{2+} they are around 10^{5} s^{-1}.[18] These are for complexes in solution and may be different in precipitates, although the relative order may remain the same. The large difference in exchange rates between Ca^{2+} and Mg^{2+} may be a reason why calcium is a better catalyst for phosphoryl transfer than magnesium. Why Fe^{2+} is so good at phosphoryl transfer is still something of a mystery.

2.3.3 The effect of temperature on the formation of pyrophosphate.

All reactions are temperature sensitive to some degree. The phosphorolysis of acetyl phosphate in aqueous solution must necessarily compete with hydrolysis and these two competing reactions will have different activation barriers and are likely to display different temperature dependencies. Temperature may also affect the exchange of phosphate and acetyl phosphate from the precipitate. Hence varying the temperature will affect the rates of phosphorolysis and hydrolysis, as well as the partitioning between the two reactions.

To investigate the effect of temperature, reaction mixtures containing 0.05M phosphate, 0.05M acetyl phosphate and 0.75M Fe^{2+} at pH 6.5 were incubated at either 22°C, 38°C, 51°C or 67°C. As expected, the rates of acetyl phosphate loss **(Figure 2.11)** and pyrophosphate formation **(Figure 2.12)** both increased as the temperature increased.

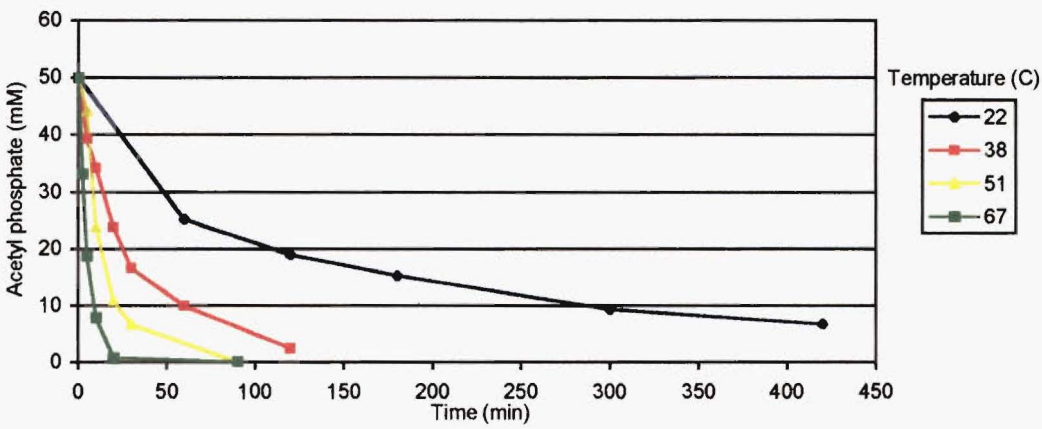

Figure 2.11. Effect of temperature on the loss of acetyl phosphate.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4 and 0.075M $FeSO_4$ were incubated at 22°C, 38°C, 51°C or 67°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

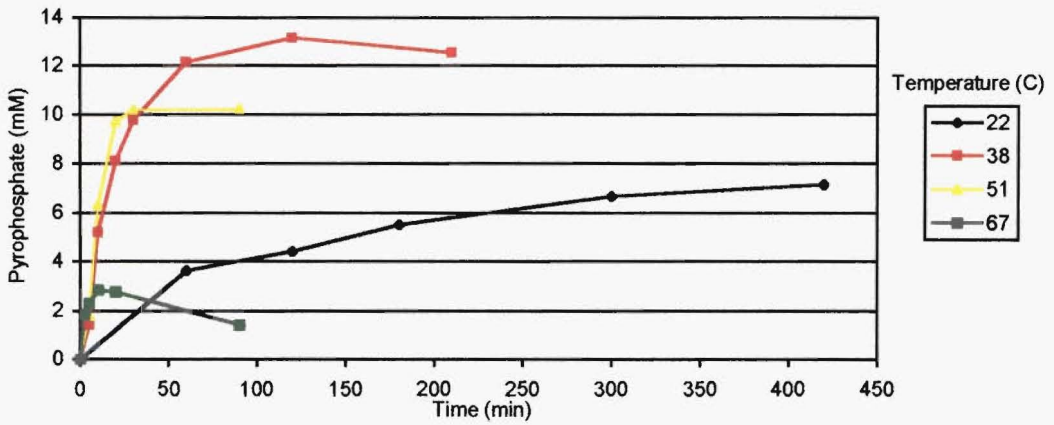

Figure 2.12. Effect of temperature on pyrophosphate formation.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4 and 0.075M $FeSO_4$ were incubated at 22°C, 38°C, 51°C or 67°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

However the yield of pyrophosphate did not remain constant, indicating the relative rates of hydrolysis and phosphorolysis did not remain constant. The maximum yield of pyrophosphate was ~25% and occurred at 38°C. The yield of pyrophosphate increased considerably going from 22°C to 38°C, and decreased considerably as the temperature was raised from 51°C to 67°C. The decrease in pyrophosphate formation going from 51°C to 67°C is a result of an greater increase in the rate of hydrolysis of acetyl phosphate relative to any increase in the rate of pyrophosphate formation, since under these conditions pyrophosphate was found to be stable over the time period of the reaction.

2.3.4 The effect of amines on the formation of pyrophosphate.

Various amines have been shown to catalyse phosphoryl transfer reactions, in particular imidazole and pyridine. A number of *N*-phosphorylated amines, for example phosphoryl imidazole and phosphoryl pyridine, have been synthesised and undergo phosphoryl transfer very readily. Herschlag and Jencks[4] reported that

pyrophosphate formation from acetyl phosphate and phosphate in the presence of sodium ions is catalysed by pyridine. Weber[19] showed that heating a solution of thioester, imidazole and phosphate produces pyrophosphate, presumably via an acetyl phosphate intermediate **(Figure 2.13)**. When imidazole was omitted from the reaction no pyrophosphate was observed.

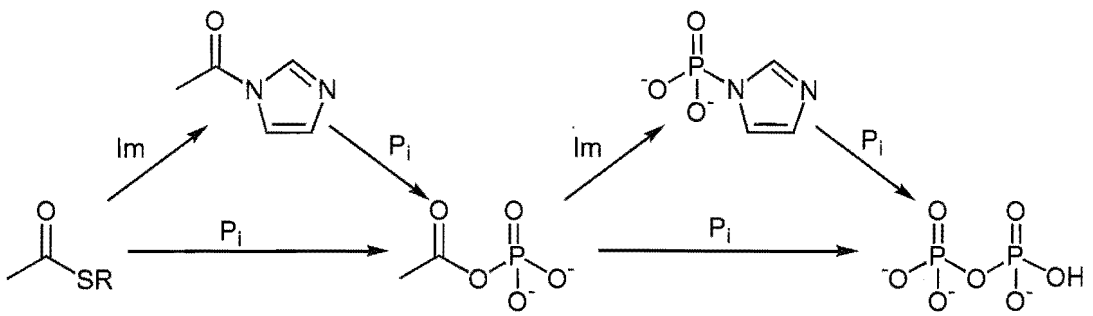

Figure 2.13. Formation of pyrophosphate from a thioester and inorganic phosphate.

Lehn[20] has synthesised a range of macrocyclic polyamines that are very efficient catalysts of phosphoryl transfer reactions. In one case an *N*-phosphorylated macrocycle was isolated, providing direct evidence that the amine attacks the acetyl phosphate at the phosphate centre **(Figure 2.14)**. These macrocycles are reported to produce pyrophosphate from acetyl phosphate and phosphate in yields of up to 40%.

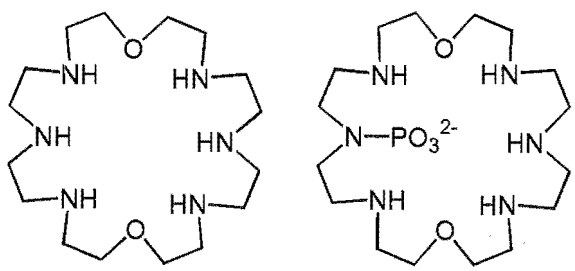

Figure 2.14. Macrocyclic polyamine and *N*-phosphorylated intermediate.

Meade[5] investigated the role of amines in phosphoryl transfer reactions of acetyl phosphate. Amines did not promote pyrophosphate formation on their own, but yields of 4% pyrophosphate were obtained in the presence of Mg^{2+} and pyridine.

Other amines did not produce pyrophosphate in the presence of Mg^{2+}. Amines are nucleophilic catalysts and may react with acetyl phosphate to form an *N*-phosphoryl amine intermediate that is more electrophilic than acetyl phosphate and reacts faster with nucleophiles **(Figure 2.15)**.

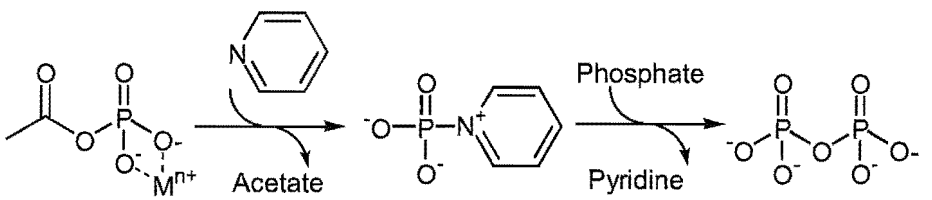

Figure 2.15. Nucleophilic catalysis of phosphoryl transfer by pyridine.

It appears that amines may be able to act in concert with metal ions to promote phosphoryl transfer, so the effect of amines of the formation of pyrophosphate in the presence of Fe^{2+} was investigated. Meade[5] had previously found that many amines, including imidazole and pyridine, did not appear to affect pyrophosphate formation in the presence of ferrous ions. However the large error limits associated with her experiments, and the wide variation in the yields of pyrophosphates, prevented her from making definitive conclusions regarding the effect of amines.

A variety of amines were chosen for the studies reported here **(Figure 2.16)**. Glycine and adenosine are both important biochemicals. Imidazole is found in the amino acid histidine, an amino acid that plays an important role in many phosphoryl transfer reactions. The imidazole ring of histidine is often a ligand for metal ions, including Fe^{2+}, in proteins as well. Pyridine has already been shown to catalyse phosphoryl transfer from acetyl phosphate.[4]

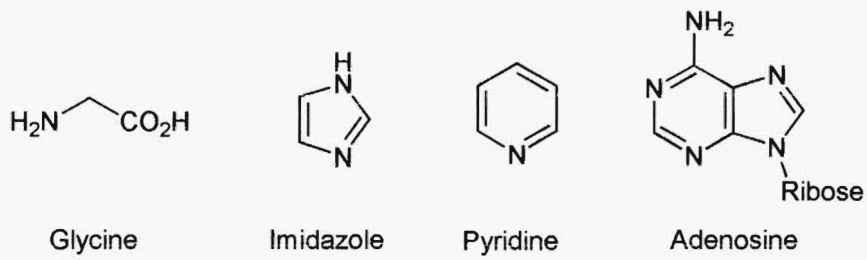

Figure 2.16.

The effect of these amines on pyrophosphate formation was determined in reactions containing 0.075M Fe^{2+} and 0.05M acetyl phosphate, pyrophosphate and amine at pH 6.5. The reactions were carried out at 38°C and at 51°C.

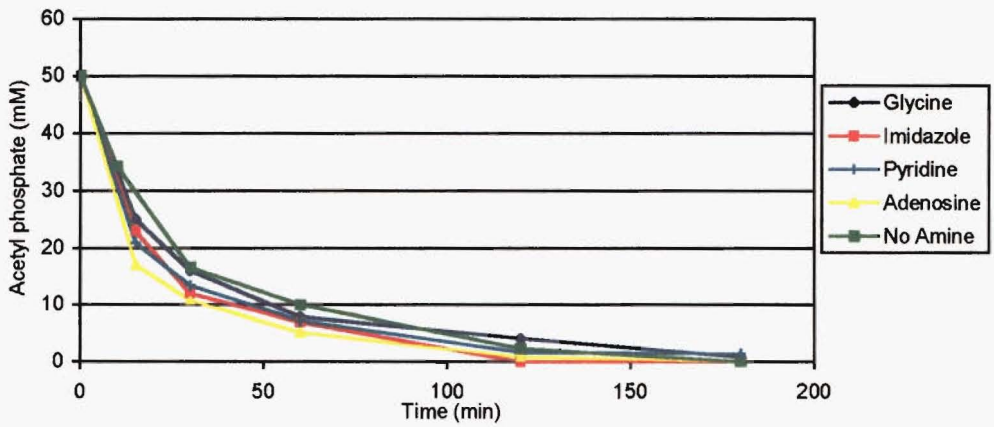

Figure 2.17. Effect of amines on the loss of acetyl phosphate at 38°C.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4, 0.05M amine and 0.75M $FeSO_4$, were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

At 38°C the various amines all had the same effect. The rate of loss of acetyl phosphate was essentially the same with or without amines **(Figure 2.17)**, while pyrophosphate formation was approximately half that obtained in the absence of amines **(Figure 2.18)**.

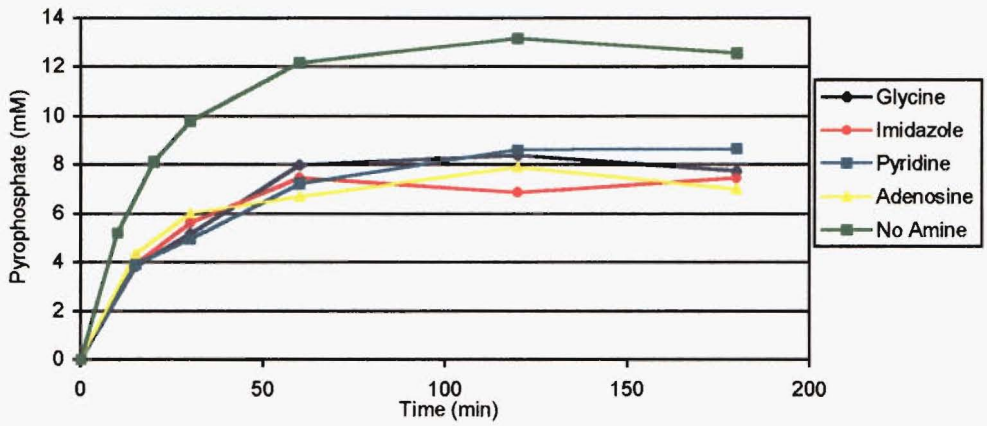

Figure 2.18. Effect of amines on pyrophosphate formation at 38°C.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4, 0.05M amine and 0.75M $FeSO_4$ were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

When the temperature was raised to 51°C, the rate of acetyl phosphate loss was slower in the presence amines than it was without amines, with the exception of glycine, which did not affect the rate of acetyl phosphate loss **(Figure 2.19)**.

At this temperature, pyrophosphate formation was much lower in the presence of the amines than it was in the absence of amines. Again, the exception was glycine, which had no effect on pyrophosphate formation **(Figure 2.20)**.

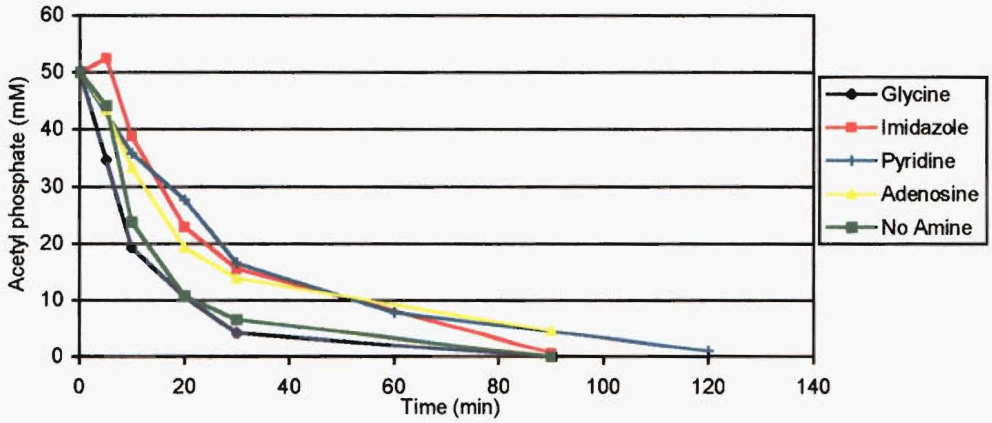

Figure 2.19. Effect of amines on the loss of acetyl phosphate at 51°C.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4, 0.05M amine and 0.75M $FeSO_4$ were incubated at 51°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

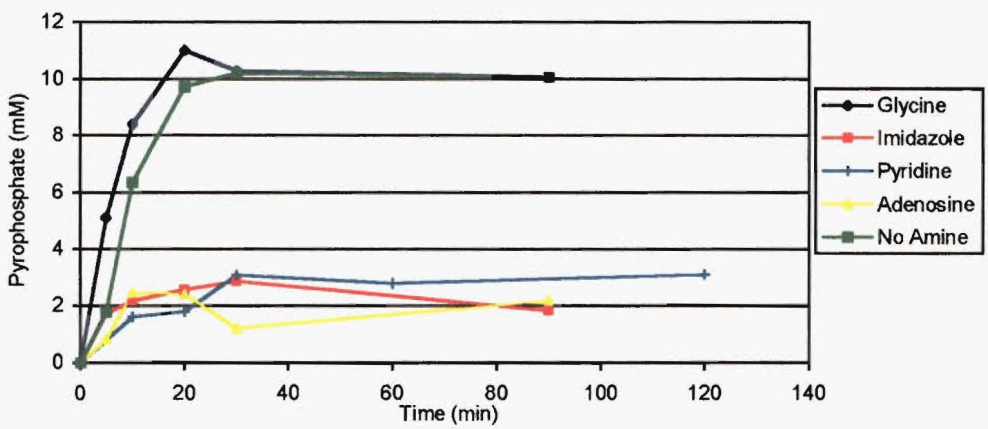

Figure 2.20. Effect of amines on pyrophosphate formation at 51°C.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4, 0.05M amines and 0.75M $FeSO_4$ were incubated at 51°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

In theory, the reduced yield of pyrophosphate could be due to three factors: an increase in the rate of hydrolysis of acetyl phosphate; a reduction in the rate of

pyrophosphate formation; or an increase in the rate of hydrolysis of pyrophosphate. The stability of pyrophosphate under these conditions was studied and no hydrolysis was observed within the time period of the experiment. The lower yield of pyrophosphate must then be due to a lower rate of phosphorolysis relative to hydrolysis of acetyl phosphate.

Amines are reported to accelerate the hydrolysis of acyl phosphates,[21] but this was not observed in these studies. At 38°C it appears that the amines affect the partitioning between hydrolysis and phosphorolysis, since the rate of acetyl phosphate loss is similar with or without amines present, while the amount of pyrophosphate formed is lower in the presence of amines. However, the experiments at 51°C suggest that imidazole, pyridine and adenosine affect both the reactivity of acetyl phosphate and the partitioning between hydrolysis and phosphorolysis, as the rates of both pyrophosphate formation and acetyl phosphate loss are lower in the presence of these amines.

How the amines influence the reactions of acetyl phosphate in the presence of iron is unclear. From the data, the amines studied do not appear to be effective nucleophilic catalysts for phosphoryl transfer under these conditions. Amines are known ligands of metal ions and may have competed with acetyl phosphate and inorganic phosphate for the Fe^{2+}. This would have the net effect of lowering the amount of Fe^{2+} available to bind the phosphate. This has already been shown to reduce the reactivity of acetyl phosphate and the rate of pyrophosphate formation. Since this work was carried out at pH 6.5, some of the amines would have been protonated, as their pK_a values range from 3.5 for adenosine to 9.6 for glycine. Glycine, in particular, would be present almost entirely as the protonated amine. This may explain why glycine had very little effect compared to the other amines. Protonated amines are ineffective nucleophilic catalysts, and are less likely to complex with metal ions, compared to unprotonated amines.

2.3.5 The effect of Fe(II)-complexing agents.

To assess whether binding of phosphates is essential for the formation of pyrophosphate, the effect of complexing the Fe(II) was investigated. The ligands chosen were cyanide and EDTA as they both bind strongly to Fe(II) but their respective complexes have differing labilities. The formation constant, β_6, for $Fe(CN)_6^{4-}$ is 10^{36} and the cyanide ligands are very inert to substitution.[22] This is because ferrocyanide is a low-spin complex and for ligand substitution to occur a change to the high-spin state is required. Hence there will be no possibility of phosphate compounds being able to bind to the Fe(II) centre. The formation constant for $Fe(EDTA)^{2-}$ is also high ($\log K_f = 16.5$).[23] However the EDTA ligand is substitutionally labile so in this case there will be an opportunity for phosphates to bind to the Fe(II).

As expected, no precipitate was observed in the reaction containing ferrocyanide, while a precipitate was observed in the reaction containing EDTA. In both cases the rate of reaction of acetyl phosphate was slower compared to the reaction with only Fe^{2+} present **(Figure 2.21)**. This is consistent with the enhanced reactivity of acetyl phosphate in the presence of Fe^{2+} being associated with an ability to bind to the metal ion.

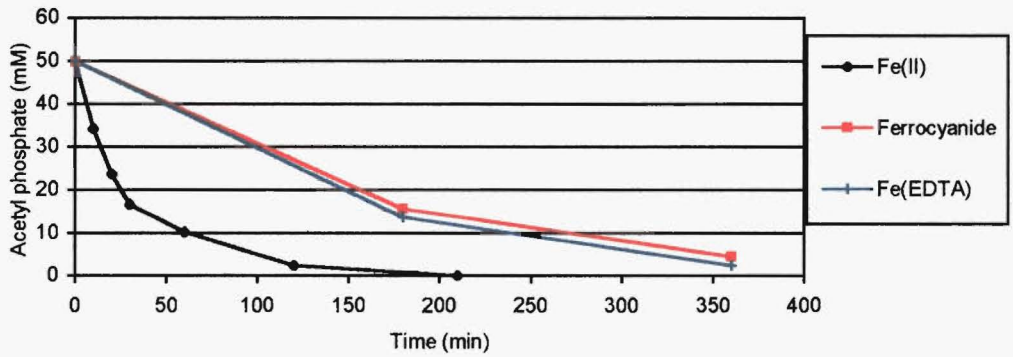

Figure 2.21. Effect of $Fe(CN)_6^{4-}$ and $Fe(EDTA)^{2-}$ on the rate of reaction of acetyl phosphate.

Buffered reactions containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4 and either 0.075M $Fe(CN)_6^{2-}$, or 0.075M $FeSO_4$ and 0.075M Na_4EDTA, were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

However, in neither case was pyrophosphate observed. The inability to form pyrophosphate in the presence of EDTA suggests that the presence of a precipitate is a necessary, but not sufficient, requirement for pyrophosphate formation. EDTA may inhibit pyrophosphate formation by co-ordinating to Fe^{2+} ions, which effectively reduces the amount of Fe^{2+} available to coordinate with phosphate species. As we have already seen, pyrophosphate formation is dependent on the relative amount of Fe^{2+}, as this affects the co-precipitation of phosphate and acetyl phosphate.

2.3.6 Pyrophosphate formation catalysed by other transition metals.

Having shown that Fe(II) is an effective catalyst for pyrophosphate formation, it is of interest to compare catalysis by ferrous ions with other related metal ions. The first row transition metals (including zinc) often display similar chemical behaviour, so given that Fe^{2+} is able to affect pyrophosphate formation, other transition metals may also. Like Fe^{2+}, the other divalent transition metals form metal-phosphate precipitates. They also form a wide variety of complexes with organic ligands, and

their complexes are generally substitutionally labile. Many of the first row transition metals are essential elements for life and would have been readily available on the early earth.

The difficulty with studying transition metals in this system has already been described for the study of Fe^{2+}; that is they undergo complex redox chemistry, form insoluble precipitates with phosphates and are often paramagnetic. However, Ni(II) and Zn(II) are relatively easily studied. Zn(II) is redox inactive and diamagnetic, while Ni(II) is not very redox active under the conditions used in this study. Both Ni(II) and Zn(II) form strong complexes with cyanide, which desorbs any phosphate species from the precipitate and prevents the metal from interfering in the NMR. Other transition metals were less amenable to study and were not investigated.

To compare the ability of Ni^{2+} and Zn^{2+} to catalyse pyrophosphate formation with catalysis by Fe^{2+}, mixtures containing 0.05M acetyl phosphate and inorganic phosphate and 0.1M metal ion at pH 6.5 were incubated at 38°C. These conditions had previously been shown to give a maximum yield of pyrophosphate in the presence of Fe^{2+}. Both the loss of acetyl phosphate **(Figure 2.22)** and the formation of pyrophosphate were monitored **(Figure 2.23)**.

The rate of acetyl phosphate loss is similar for Zn^{2+} and Fe^{2+}, but is slower for Ni^{2+}. However, for Ni^{2+} and Zn^{2+} the rate of pyrophosphate formation was almost identical, with a yield of ~4%. This compares to a yield of pyrophosphate in the presence of Fe^{2+} of ~25%. The differences in the rate of acetyl phosphate loss for Ni^{2+} and Zn^{2+} suggest that these two metal ions influence different aspects of pyrophosphate formation.

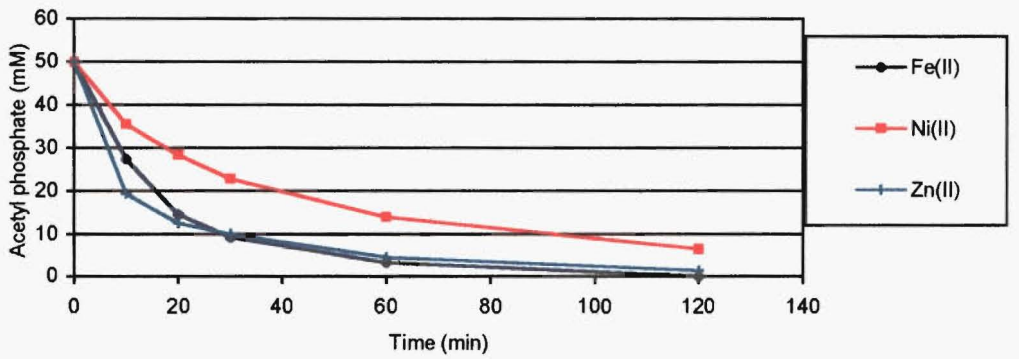

Figure 2.22. Effect of metal ions on the loss of acetyl phosphate.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4 and 0.1M $FeSO_4$, $NiSO_4$ or $ZnSO_4$, were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

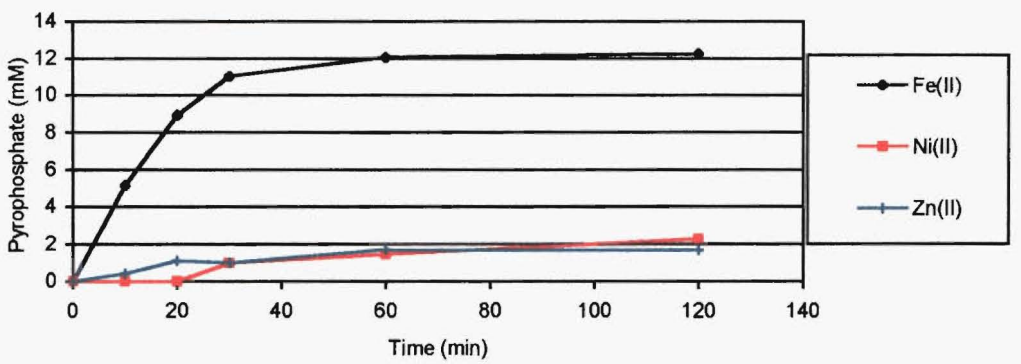

Figure 2.23. Effect of metal ions on pyrophosphate formation.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4 and 0.1M $FeSO_4$, $NiSO_4$ or $ZnSO_4$, were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

The rate of acetyl phosphate loss is similar in the presence of Fe^{2+} and Zn^{2+} even though the rate of pyrophosphate formation is less with Zn^{2+} present. This indicates that Zn^{2+} increases the rate of acetyl phosphate hydrolysis relative to pyrophosphate formation. This is consistent with Zn^{2+} being a good Lewis acid. It is often found in hydrolytic enzymes in biology, particularly when the substrates are small or when the hydrolysis is indiscriminate.

The rates of both acetyl phosphate loss and of pyrophosphate formation are lower in the presence of Ni^{2+} compared with Fe^{2+}. Since the difference in the rate of acetyl phosphate loss in the presence of Ni^{2+} compared to Fe^{2+} is not very large, it may be due to the different rates of phosphorolysis in the presence of these two ions. Why Fe^{2+} is a better catalyst for phosphoryl transfer than Ni^{2+} is unclear, as the two ions are fairly similar. One of the few differences between the two ions is in their rates of ligand substitution. Exchange rates for Fe^{2+} are on the order of 10^6 s^{-1}, while for Ni^{2+} the rates are ~10^4 s^{-1} in solution. Since the reaction of acetyl phosphate and phosphate presumably takes place on the metal ion, the rate of exchange is likely to effect the rate of reaction, with faster exchange rates leading to faster reaction rates.

2.4 Systems with Biological Relevance.

The work described in the previous section demonstrated that Fe^{2+} is an efficient catalyst for the formation of pyrophosphate from acetyl phosphate and inorganic phosphate. This section adapts the chemistry that has been described to systems that are more biologically relevant. Phosphoryl transfer in the presence of FeS is described first, followed by a discussion of phosphoryl transfer to other phosphate nucleophiles, such as ADP.

2.4.1 Pyrophosphate formation catalysed by FeS.

As mentioned in chapter one, a major source of Fe(II) on the early earth was in the mineral ferrous sulfide. Phosphate is often found as a minor component in many minerals, including FeS.[9] Wächterschäuser has postulated a central role for FeS in the origins of metabolism.[24] The importance of iron sulfides in the evolution of life may be reflected in the continued use of Fe/S clusters as protein co-factors in biochemistry. It was of interest to see whether FeS was able to catalyse phosphoryl transfer in the same way that Fe^{2+} can. FeS might be expected to be less effective as a catalyst than Fe^{2+} since the sulfide competes with phosphate species for binding sites.

Ferrous sulfide was generated *in situ* by adding a solution of $FeSO_4$ to a solution that contained NaSH, as well as phosphate and acetyl phosphate. A very fine black precipitate, the appearance of which was consistent with FeS, formed immediately. The phosphoryl transfer reaction was monitored in mixtures containing 0.1M Fe^{2+} and S^{2-} and 0.05M acetyl phosphate and phosphate at a pH of 6.5. Aliquots of the reaction were treated with potassium cyanide to desorb phosphates from the mineral surface prior to analysis. The loss of acetyl phosphate in the presence of FeS or Fe^{2+} is shown below **(Figure 2.24)**. Similarly, the formation of pyrophosphate in the presence of FeS compared with pyrophosphate formation in the presence of Fe^{2+} is also shown **(Figure 2.25)**.

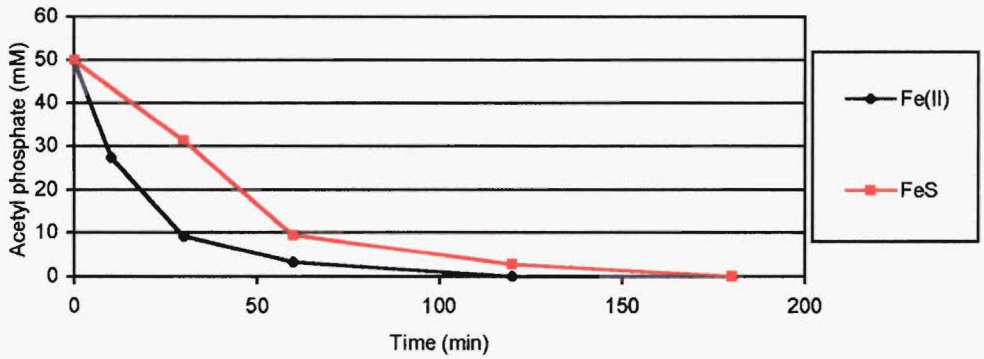

Figure 2.24. Acetyl phosphate loss in the presence of Fe^{2+} or FeS.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4 and 0.1M $FeSO_4$ or 0.1M $FeSO_4$ and 0.1M NaSH were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

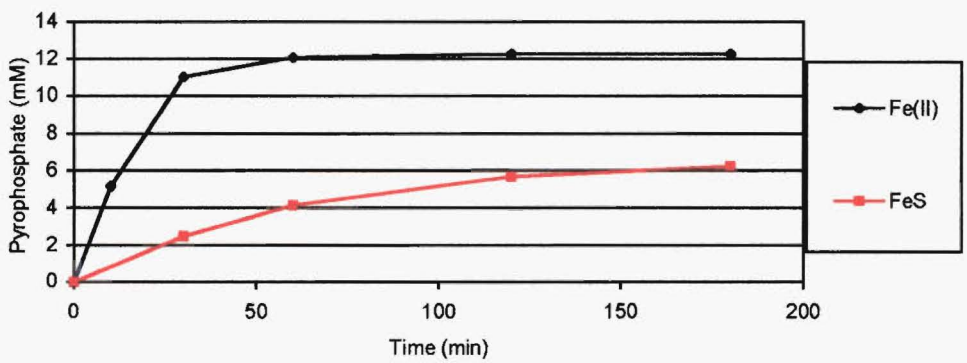

Figure 2.25. Pyrophosphate formation in the presence of Fe^{2+} or FeS.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M K_2HPO_4 and 0.1M $FeSO_4$ or 0.1M $FeSO_4$ and 0.1M NaSH were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

The rate of acetyl phosphate loss is slower in the presence of FeS then in the presence of Fe^{2+}. This is consistent with FeS having fewer sites able to bind phosphate species and catalyse the reaction of acetyl phosphate. However, these sites

are capable of catalysing phosphoryl transfer to inorganic phosphate, as the yield of pyrophosphate in the presence of 0.1M FeS was 12%, about half the yield obtained in the presence of 0.1M Fe^{2+}.

While the yield of pyrophosphate is lower in the presence of FeS than with Fe^{2+}, the yield is still much higher than in the presence of either Ni^{2+} or Zn^{2+}. FeS could provide Fe^{2+} centres for the catalysis of pyrophosphate formation in two ways. The surface of FeS contains positively charged centres and is able to bind anionic ligands. FeS may catalyse pyrophosphate formation by providing Fe^{2+} sites on the surface on the mineral that are able to bind phosphate and acetyl phosphate, allowing reaction to take place (**Figure 2.26**). Alternatively, phosphate may displace sulfide from the FeS, forming co-precipitates that include ferrous phosphates, which may be the site of pyrophosphate formation.

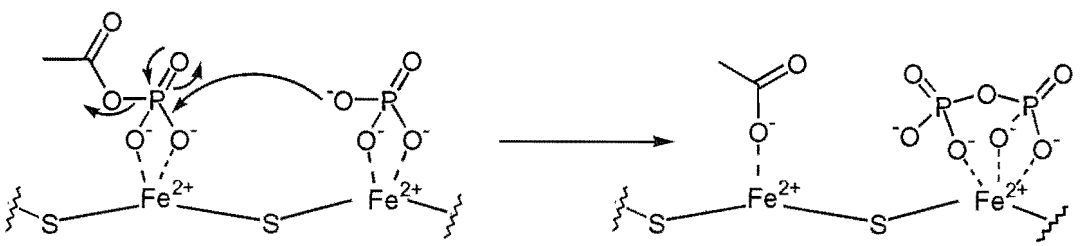

Figure 2.26. Schematic view of pyrophosphate formation catalysed by FeS.

2.4.2 Phosphoryl transfer to acceptors other than phosphate.

Having shown that Fe^{2+} is an efficient catalyst for the formation of pyrophosphate from acetyl phosphate and phosphate, it was of interest to see if phosphoryl transfer could take place with acceptors other than inorganic phosphate. The obvious candidates as acceptors are organic phosphates, such as AMP and ADP. Nucleoside diphosphates, like ADP, are the usual phosphoryl acceptors in biochemistry. Another potential phosphoryl acceptor is pyrophosphate itself; this would form tripolyphosphate, which is identical to the triphosphate moiety of ATP.

The only reported system that has been shown to catalyse phosphoryl transfer from acetyl phosphate to an acceptor other than phosphate are the macrocyclic polyamines of Lehn.[25] These macrocycles catalyse the phosphorylation of ADP by acetyl phosphate, producing ATP. Interestingly the production of ATP is stimulated by Mg^{2+} ions. ATP was formed in 16% yield in the absence of Mg^{2+}, and in 26% yield in the presence of Mg^{2+}. In addition, the macrocycles catalyse the formation of a small amount (ca. 2%) of tripolyphosphate from acetyl phosphate and pyrophosphate.[20] However AMP and glucose-1-phosphate were not phosphorylated under similar conditions.

The ability of Fe^{2+} to catalyse phosphoryl transfer from acetyl phosphate to AMP, ADP or pyrophosphate was studied under the conditions that gave the greatest yield of pyrophosphate formation. Mixtures containing 0.075M Fe^{2+}, 0.05M acetyl phosphate and 0.05M phosphoryl acceptor at pH 6.5 were incubated at 38°C. The reactions were followed by withdrawing samples at various times, treating them with potassium cyanide, and analysing them using ^{31}P NMR. Somewhat disappointingly no phosphoryl transfer to AMP, ADP or pyrophosphate was observed. The rate of acetyl phosphate loss with AMP, ADP or pyrophosphate as the phosphoryl acceptor was slower than when phosphate was the phosphoryl acceptor **(Figure 2.27)**.

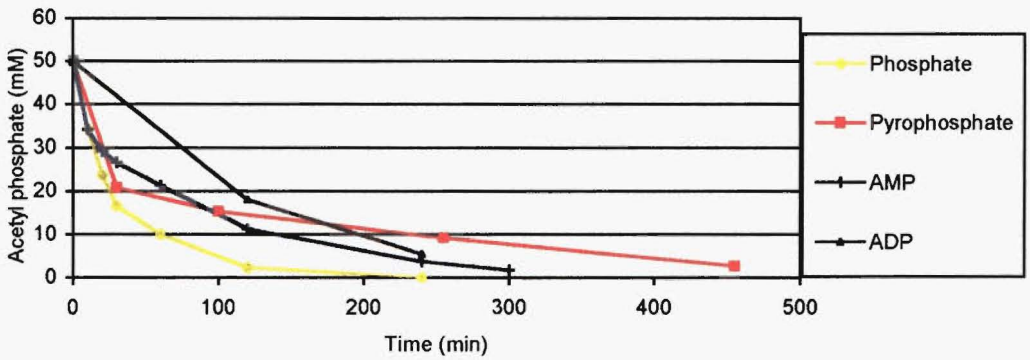

Figure 2.27. Acetyl phosphate loss with different phosphoryl acceptors.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M phosphoryl acceptor and 0.075M $FeSO_4$ were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

The slower rate of acetyl phosphate loss when phosphoryl acceptors other than inorganic phosphate are used is probably due to the low rate of phosphorolysis. The inability of acetyl phosphate to phosphorylate AMP, ADP or pyrophosphate may be due to the types of complexes these phosphates form with Fe^{2+}, or to changes in the precipitates formed. Pyrophosphate, AMP and ADP all have the ability to form chelates with Fe^{2+}, and this may prevent the co-precipitation of acetyl phosphate and the phosphoryl acceptor required for phosphoryl transfer from occurring. The types of complexes these phosphates form with Fe^{2+} is described in more detail in chapter 4. A precipitate containing AMP, ADP or pyrophosphate is also likely to be considerably different than a precipitate consisting mainly of phosphate. This is especially true for AMP and ADP which have fairly large hydrophobic groups attached to them. It may be that a ferrous phosphate precipitate has surface sites that are able to bind acetyl phosphate and promote phosphorylation. These sites may be lacking in precipitates with AMP, ADP or pyrophosphate.

In the reaction containing AMP, traces of pyrophosphate appeared some time after the reaction was initiated **(Figure 2.28)**.

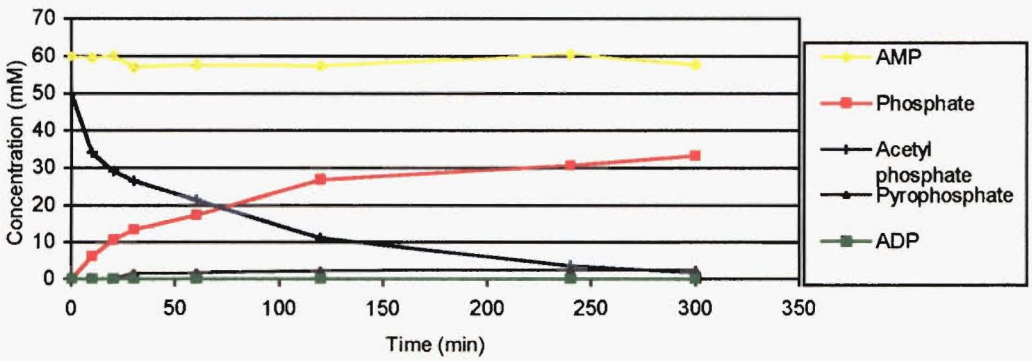

Figure 2.28. Attempted phosphorylation of AMP by acetyl phosphate.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.06M AMP and 0.075M $FeSO_4$ was incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

Pyrophosphate formation begins after a short lag time, suggesting that the inorganic phosphate required for pyrophosphate formation comes from the hydrolysis of acetyl phosphate. The formation of pyrophosphate was not observed in the reactions containing either ADP or pyrophosphate as the phosphoryl acceptor. In the case when pyrophosphate was present as the phosphoryl acceptor, it is difficult to infer the formation of additional pyrophosphate.

To see if a ferrous phosphate co-precipitate is required for the phosphorylation of AMP by acetyl phosphate, phosphate was added to the reaction. The rate of acetyl phosphate loss is slower in the presence of AMP, compared to the reaction without AMP **(Figure 2.29)**.

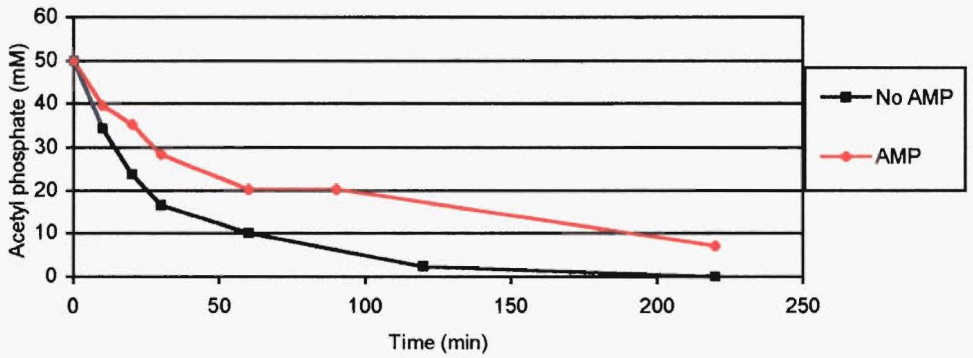

Figure 2.29. Loss of acetyl phosphate in the presence of AMP.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M phosphate and 0.075M Fe^{2+} were incubated with or without 0.05M AMP at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

AMP is capable of co-ordinating with metal ions and will compete with acetyl phosphate for ferrous ions. Since the rate of reaction of acetyl phosphate is dependent on the amount of Fe^{2+} present, the presence of AMP results in a decrease the rate of reaction of acetyl phosphate. Again no ADP was observed, although a substantial amount of pyrophosphate was formed **(Figure 2.30)**.

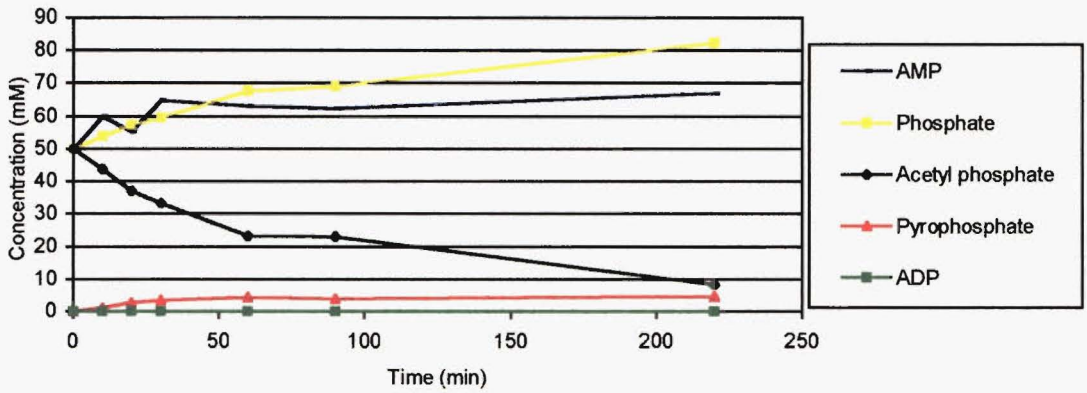

Figure 2.30. Phosphoryl transfer in the presence of AMP and phosphate.

Buffered mixtures containing 0.05M $CH_3CO_2PO_3Li_2$, 0.05M phosphate, 0.05M AMP and 0.075M Fe^{2+} were incubated at 38°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with KCN.

The amount of pyrophosphate formed from acetyl phosphate and phosphate in the presence of AMP was less than half that formed in the absence of AMP (9.2% vs. 25%). Again, since pyrophosphate formation is dependent on the amount of Fe^{2+} present, this is most likely due to AMP competing with acetyl phosphate for Fe^{2+}. AMP also contains the adenosine moiety, which was previously shown to reduce the formation of pyrophosphate considerably.

2.5 Chapter Summary.

The work described in this chapter demonstrates that Fe^{2+} is a very efficient catalyst for the formation of pyrophosphate from acetyl phosphate and inorganic phosphate. The reaction was found to be pH dependent, and a maximum yield of pyrophosphate was obtained at pH 6.5. This is possibly due to an increase in the binding of phosphates to Fe^{2+} as the pH is raised. The yield of pyrophosphate decreased above a pH of ~7, possibly due to the formation of insoluble iron hydroxides.

The formation of pyrophosphate was also dependent on the relative amount of Fe^{2+} present. This is possibly due to changes in the composition of the precipitate, since at low levels of Fe^{2+}, selective precipitation of phosphate will occur.

Pyrophosphate formation is temperature dependent, and a maximum yield of pyrophosphate was obtained at a temperature of 38°C. The temperature dependence of pyrophosphate formation is due to changes in the relative rates of hydrolysis and phosphorolysis. The pH and temperature dependencies of pyrophosphate formation resemble the characteristics of many enzyme catalysed reactions.

In the presence of Fe^{2+}, some amines were shown to have an effect on the formation of pyrophosphate. At 38°C, in the presence of glycine, imidazole, pyridine or adenosine, pyrophosphate formation was half that in the absence of any amines. The yield of pyrophosphate in the presence of the same amines was reduced even further at 51°C, with the exception of glycine, which appeared to have no effect on pyrophosphate formation. The reasons for this are unclear, although it appears that amines interfere with the phosphorolysis reaction and had a negligible effect on the hydrolysis of acetyl phosphate. The amines did not catalyse the hydrolysis of pyrophosphate under these conditions.

The formation of pyrophosphate appears to take place in, or on the surface of, a mixed precipitate containing Fe^{2+}, phosphate and acetyl phosphate. The precipitate is essential as ligands that bind to Fe^{2+} and prevent the formation of a precipitate have a detrimental effect on pyrophosphate formation. No pyrophosphate formation was observed when EDTA was added to the reaction, or when the Fe^{2+} was complexed with cyanide ions.

Pyrophosphate formation is also catalysed by Ni^{2+} and Zn^{2+}, although the yields of pyrophosphate are much lower than when Fe^{2+} is present. Ni^{2+} and Zn^{2+} appear to influence different aspects of the pyrophosphate formation. Ni^{2+} affects the rate of

phosphorolysis while Zn^{2+} appears to affect the partitioning between the hydrolysis and phosphorolysis of acetyl phosphate.

FeS was also shown to catalyse the formation of pyrophosphate, even though the sulfide competes with the phosphate for the Fe^{2+} centres. The ability of FeS to catalyse this reaction may have been important during the origin of life, as FeS was present in vast quantities during this time.

Phosphoryl transfer from acetyl phosphate to phosphate nucleophiles other than inorganic phosphate was not observed under the conditions used. This may have been due to changes in the nature of the precipitates formed. In some cases pyrophosphate formation was still observed, as a result of the release of inorganic phosphate during the hydrolysis of acetyl phosphate. The inability to observe phosphoryl transfer was not due to the instability of the expected products, as these were stable over the time frame of the experiments.

The ability of Fe^{2+} to catalyse the formation of pyrophosphate from acetyl phosphate and inorganic phosphate warrants further investigation. The structure of the ferrous phosphate precipitate remains to be elucidated as well as the site of reaction. Does reaction occur on the surface of a precipitate consisting of mainly Fe^{2+} and inorganic phosphate, or does it occur within the precipitate with acetyl phosphate and phosphate distributed evenly throughout the precipitate. Perhaps most importantly, can phosphoryl transfer to other phosphate nucleophiles be demonstrated?

2.6 References for Chapter Two.

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3 Phosphoenolpyruvate Chemistry.

3.1 Introduction.

3.1.1 Phosphoenolpyruvate in biology.

Having shown in the previous chapter that Fe^{2+} is an excellent catalyst for the formation of pyrophosphate from acetyl phosphate and phosphate, it was of interest to see if Fe^{2+} could catalyse phosphoryl transfer from other high-energy phosphate species. Another biologically important phosphoryl donor is phosphoenolpyruvate, which is formed during glycolysis. Phosphoenolpyruvate phosphorylates ADP, generating ATP and pyruvate in one of the two ATP generating reactions that occur during glycolysis **(Figure 3.1)**.

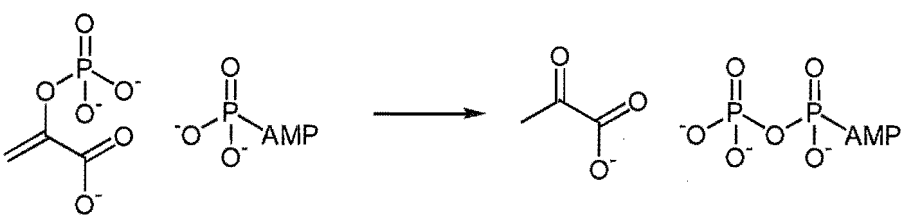
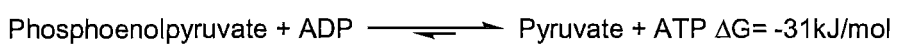


Figure 3.1. Generation of ATP from phosphoenolpyruvate and ADP.

The other substrate-level phosphorylation that occurs in glycolysis is the generation of ATP by the reaction of ADP with 1,3-diphosphoglycerate, an acyl phosphate. The ability of another acyl phosphate, acetyl phosphate, to act as a phosphoryl donor was the subject of the previous chapter.

3.1.2 Thermodynamics of phosphoryl transfer from phosphoenolpyruvate.

Phosphoenolpyruvate is an energy-rich compound, with a free energy of hydrolysis of -62.0 kJ/mole.[1] This is an even higher free energy of hydrolysis than acyl phosphates, and represents an equilibrium constant of ~10^{10} in favour of hydrolysis to pyruvate and phosphate. Since the free energy of hydrolysis of a phosphoanhydride bond is ~ -31 kJ/mole, the thermodynamics of a phosphoryl-group transfer between phosphoenolpyruvate and ADP is very favourable. The equilibrium constant for the biological reaction is ~3000, making the formation of ATP essentially irreversible.



Why is the phosphoryl-group-transfer potential of phosphoenolpyruvate so high? Unlike acyl phosphates, which are mixed anhydrides, the structure of phosphoenolpyruvate does not appear to be particularly unstable. The answer lies in the structures of the products of hydrolysis. The initial products of hydrolysis of phosphoenolpyruvate are phosphate and the enol form of pyruvate. However, the enol form of pyruvate does not exist free in solution in significant concentrations, it rapidly tautomerises to the keto form. The hydrolysis of phosphoenolpyruvate can be thought of as occurring in two steps. The hydrolysis to inorganic phosphate and the enol form of pyruvate contributes –16kJ/mole and the tautomerism to the keto form of pyruvate contributes a further –46kJ/mole **(Figure 3.2)**.

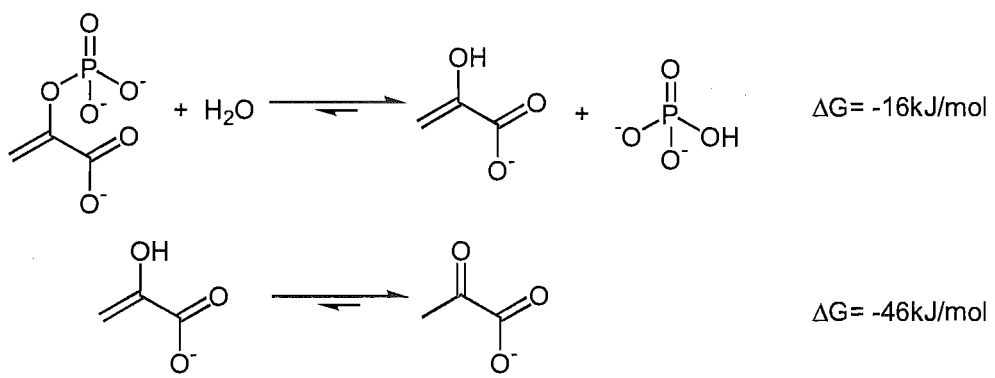


Figure 3.2. Contributions to the free energy of hydrolysis of phosphoenolpyruvate.

3.1.3 Fe(II) catalysis of phosphoryl transfer from phosphoenolpyruvate.

Looking at the enzymatic reaction supports the notion that Fe^{2+} may be capable of catalysing phosphorylation reactions using phosphoenolpyruvate as the phosphoryl-group donor. The formation of ATP from phosphoenolpyruvate and ADP is catalysed *in vivo* by the enzyme pyruvate kinase. Pyruvate kinase has a requirement for a divalent metal cation, usually magnesium.[2] A direct transfer of the phosphoryl group from phosphoenolpyruvate to ADP occurs; no phosphoenzyme intermediate, such as phosphorylated histidine, has been detected. This suggests that the role of the enzyme is to correctly orientate the substrates to allow the reaction to occur. A ferrous-phosphate precipitate could carry out an analogous orientation of phosphoenolpyruvate and phosphate to allow pyrophosphate formation to take place. The precipitate may also serve to concentrate the reactants and reduce unfavourable electrostatic interactions between the phosphoryl-group and the phosphate.

Hermes-Lima and Vieyra[3] have previously shown that formation of pyrophosphate from phosphoenolpyruvate and phosphate is catalysed by magnesium and calcium precipitates. They found that pyrophosphate formation was very low in purely aqueous solution, with a yield of ~0.04%. When the reaction was performed in 80% DMSO a yield of ~5.8% pyrophosphate was obtained. DMSO promotes the desolvation of anions and decreases the activity of water. Since pyrophosphate formation necessarily competes with hydrolysis this is a useful way of reducing the rate of hydrolysis.

Preliminary experiments indicated that Fe^{2+} was an effective catalyst for the formation of pyrophosphate from phosphoenolpyruvate and phosphate. Essentially the experimental conditions were identical to those already used in the study of pyrophosphate formation using acetyl phosphate as the phosphoryl donor. Cyanide was used to desorb phosphate compounds from the Fe^{2+} at the end of the reaction.

The concentrations of phosphate containing compounds were determined using ^{31}P NMR. The only differences were that the reactions required incubation for up to 12 days, whereas the reactions involving acetyl phosphate were complete within a matter of hours. This is not surprising, as acetyl phosphate is a much more reactive compound. To prevent oxidation of Fe^{2+} from occurring over this time period the reactions were carried out in sealed vials under an atmosphere of argon and incubated in sealed chambers filled with argon.

3.2 Pyrophosphate Formation from Phosphoenolpyruvate and Phosphate.

3.2.1 The effect of pH on the formation of pyrophosphate.

The pH of the solution is likely to have a significant effect on the rate of pyrophosphate formation from phosphoenolpyruvate and phosphate. Phosphate and phosphoenolpyruvate have dissociable protons and the degree of ionisation of these will affect the strength of binding to the Fe^{2+}. The degree of ionisation will also affect the electrostatic interaction between the negatively charged phosphates of phosphoenolpyruvate and phosphate. Fe^{2+} also displays pH-dependent behaviour, and forms insoluble ferrous or ferric hydroxide precipitates above a pH of 7.

The pH-dependence of pyrophosphate formation from phosphoenolpyruvate in the presence of a calcium phosphate precipitate has been investigated by Hermes-Lima and Vieyra.[3] They found that maximum pyrophosphate formation occurred at pH 7.5. This also corresponded to the pH at which the maximum amount of phosphoenolpyruvate was adsorbed onto the precipitate. Below this pH very little Ca^{2+}-phosphate precipitate had formed and, since a precipitate was required for pyrophosphate formation, this may explain the lack of pyrophosphate formation below pH 7. They suggested that the decrease in the amount of adsorbed phosphoenolpyruvate and pyrophosphate formation as the pH increases above 7.5

may be due to modifications in the charge of the surface of the precipitate. As the pH increases, hydroxide anions may begin to compete with phosphoenolpyruvate for binding sites on the surface of the precipitate. The hydrolysis of phosphoenolpyruvate may also increase as the concentration of hydroxide increases.

Hermes-Lima and Vieyra[3] also found that by replacing the water with 80% DMSO, the amount of adsorbed phosphoenolpyruvate and pyrophosphate formation increased with pH and reached maximum values above pH 9. Since the activity of water is reduced significantly in 80% DMSO, the increase in pyrophosphate formation and adsorbed phosphoenolpyruvate may be because of a lack of competition with hydroxide. The structure of the calcium phosphate precipitate is also likely to be somewhat different in 80% DMSO compared to a purely aqueous environment.

The effect of pH on the formation of pyrophosphate was investigated over the pH range 6 to 7.5. The buffer used at pH 6 and 6.5 was MES, while MOPS was used to buffer at pH 7 and 7.5. These buffers were used because they do not interact significantly with metal ions.[4] Mixtures containing 0.05M phosphoenolpyruvate, 0.05M phosphate and 0.1M ferrous sulfate where incubated at 45°C for up to 10 days. Both the loss of phosphoenolpyruvate **(Figure 3.3)** and the formation of pyrophosphate **(Figure 3.4)** were monitored.

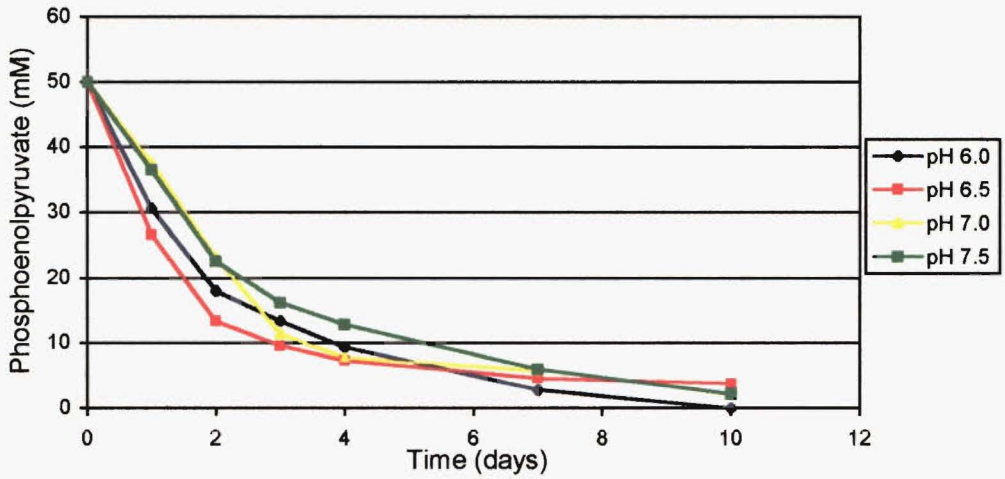


Figure 3.3. The effect of pH on the loss of phosphoenolpyruvate.

Buffered mixtures containing 0.05M phosphate, 0.05M phosphoenolpyruvate and 0.1M ferrous sulfate were incubated at 45°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with potassium cyanide.

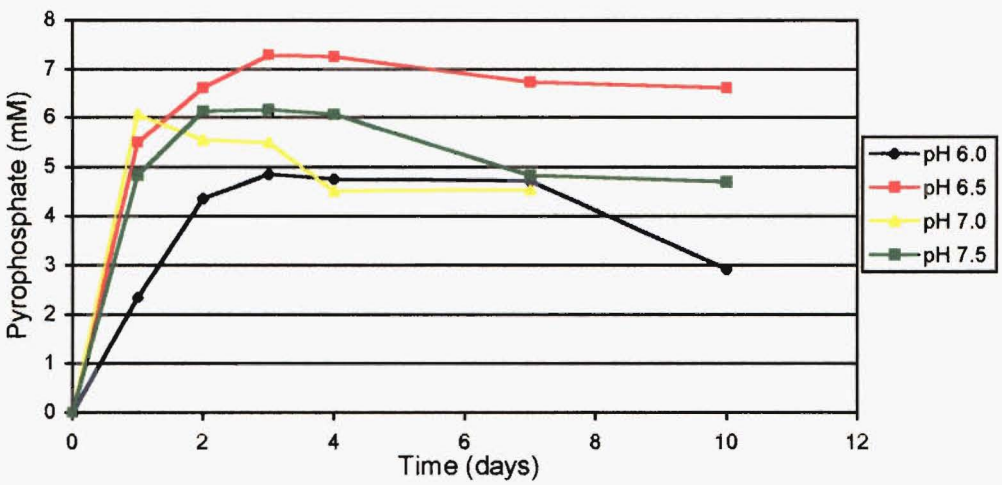


Figure 3.4. The effect of pH on the formation of pyrophosphate.

Buffered mixtures containing 0.05M phosphate, 0.05M phosphoenolpyruvate and 0.1M ferrous sulfate were incubated at 45°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with potassium cyanide.

The rate of loss of phosphoenolpyruvate did not seem to be greatly affected by the change in pH. In contrast, pyrophosphate formation was pH dependent, with the maximum yield of pyrophosphate, about 7%, occurring at pH 6.5. Above pH 6.5 the yield dropped slightly to about 6%, while the yield of pyrophosphate at pH 6 was only 4%. This is similar to the pH dependence of pyrophosphate formation from acetyl phosphate and phosphate, which also occurred in maximum yield at pH 6.5, as was described in the previous chapter.

The same factors may be invoked to explain this dependence as were described in the previous chapter. The formation of pyrophosphate is probably dependent on the amount of precipitate present. The degree of precipitation and the strength of binding of phosphates to Fe^{2+} are likely to increase with pH due to a greater degree of ionisation of the phosphates. However, as the pH increases above 7, insoluble iron hydroxide precipitates begin to form and have the effect of reducing the amount of Fe^{2+} available to bind phosphates. This may be the reason for the pH behaviour of pyrophosphate formation.

3.2.2 The effect of the concentration of Fe^{2+} on pyrophosphate formation.

The amount of Fe^{2+} in the reaction has already been shown to be an important factor in the catalysis of phosphoryl transfer from acetyl phosphate by ferrous phosphate precipitates. A probable reason for this was that the amount of precipitate and the amount of adsorbed acetyl phosphate are both likely to increase with increasing Fe^{2+}.

Hermes-Lima and Vieyra[3] found that the formation of pyrophosphate from phosphoenolpyruvate and a calcium phosphate precipitate depended on the amount of Ca^{2+} present in the reaction. Pyrophosphate formation showed a sigmoidal dependence on the concentration of Ca^{2+}, indicating that multiple metal ions are involved in the phosphoryl transfer reaction.

Catalysis of phosphoryl transfer from phosphoenolpyruvate by Fe^{2+} might also be expected to display a dependence on the amount of Fe^{2+} present. To see if this was the case, the effect of varying the concentration of Fe^{2+} over the range 0-0.1M was investigated. Mixtures containing 0.05M phosphoenolpyruvate and 0.05M phosphate and ferrous sulfate were incubated at 45°C for a number of days. The reactions were buffered at pH 6.5, as this was the pH at which the maximum yield of pyrophosphate was observed. The effect of increasing the amount of Fe^{2+} available on the rate of phosphoenolpyruvate loss **(Figure 3.5)** and the rate of pyrophosphate formation **(Figure 3.6)** are shown below.

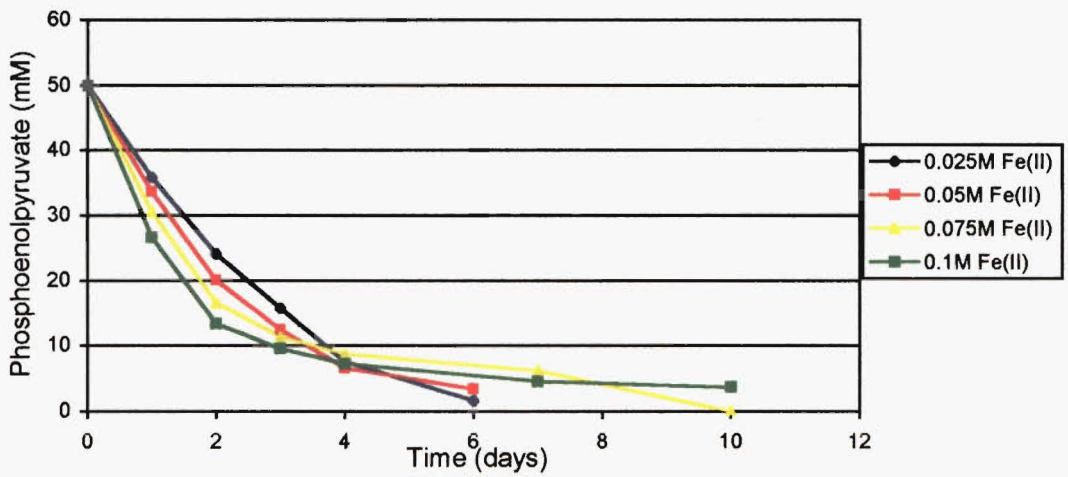


Figure 3.5. The effect of [Fe^{2+}] on the loss of phosphoenolpyruvate.

Buffered solutions containing 0.05M phosphate, 0.05M phosphoenolpyruvate and ferrous sulfate were incubated at 45°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with potassium cyanide.

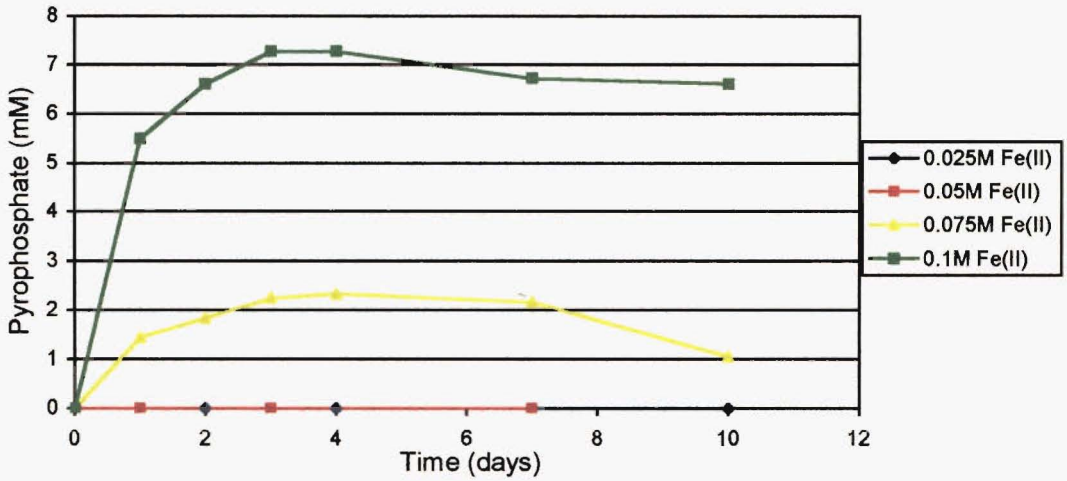


Figure 3.6. The effect of [Fe^{2+}] on the formation of pyrophosphate.

Buffered mixtures containing 0.05M phosphate, 0.05M phosphoenolpyruvate and ferrous sulfate were incubated at 45°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with potassium cyanide.

The rate of phosphoenolpyruvate loss increased as the amount of Fe^{2+} available was increased. This would be partly due to the faster rate of phosphorolysis that occurs at higher Fe^{2+} concentrations. The rate of phosphoenolpyruvate hydrolysis may also increase as the amount of Fe^{2+} present is increased. This is supported by the faster rate of phosphoenolpyruvate loss when the initial concentration of Fe^{2+} was 0.05M compared to when the initial concentration of Fe^{2+} was 0.025M. Since no pyrophosphate was observed in either case, any differences in the rate of phosphoenolpyruvate loss must be due to hydrolysis. This indicates that the reactivity of phosphoenolpyruvate towards nucleophiles is enhanced by binding to Fe^{2+}.

Pyrophosphate formation was not observed until the initial concentration of Fe^{2+} was greater than 0.05M. At a Fe^{2+} concentration of 0.075M the yield of pyrophosphate was 4.5%, this increased to 14% at an initial concentration of 0.1M ferrous ion. Increasing the amount of Fe^{2+} results in an increase in the amount of pyrophosphate formed, as well as the amount of precipitate formed. This suggests that

pyrophosphate formation occurs on or within the precipitate, and is consistent with results obtained with pyrophosphate formation from acetyl phosphate. As was the case with acetyl phosphate, selective precipitation between phosphate and phosphoenolpyruvate will occur when the amount of Fe^{2+} is limiting. Inorganic phosphate will precipitate first at low levels of Fe^{2+}, as it binds more strongly to Fe^{2+}. As the amount of Fe^{2+} available increases, the amount of phosphoenolpyruvate in the precipitate will increase, since most of the phosphate will have already precipitated.

Pyrophosphate formation from phosphoenolpyruvate requires a higher level of Fe^{2+} compared to pyrophosphate formation from acetyl phosphate. Why this is the case is unclear. It may be that phosphoenolpyruvate does not bind as tightly to Fe^{2+} as acetyl phosphate does. However, very little is known about the association constants of acetyl phosphate and phosphoenolpyruvate with divalent metal ions, let alone with Fe^{2+}. The precipitate may catalyse the formation of pyrophosphate from phosphoenolpyruvate and inorganic phosphate in a variety of ways. The precipitate may bring the substrates in close proximity to one another, as well as positioning them in the correct orientation for phosphoryl transfer to occur **(Figure 3.7)**. The ferrous ions will also reduce the electrostatic repulsion of the phosphate groups as they are brought close together by reducing some of the electron density.

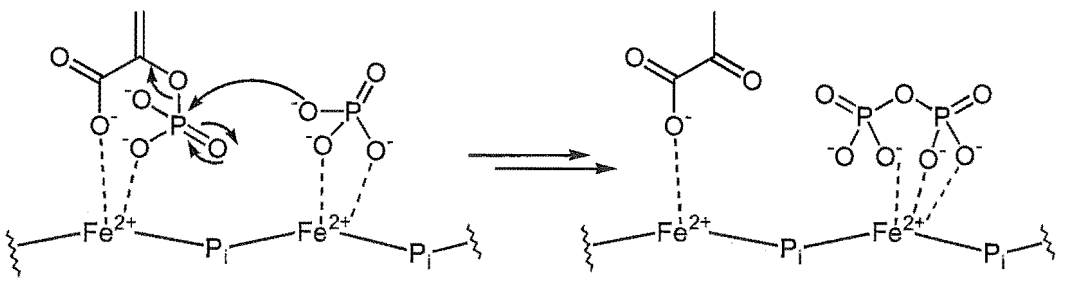


Figure 3.7. Formation of pyrophosphate from phosphoenolpyruvate and phosphate.

3.2.3 Phosphorylation of AMP and ADP by phosphoenolpyruvate.

The previous sections examined the effect pH and Fe^{2+} concentration had on the rate of pyrophosphate formation from phosphoenolpyruvate and inorganic phosphate. Having shown that phosphoryl transfer to inorganic phosphate can occur, it is of interest to see if Fe^{2+} can catalyse phosphoryl transfer from phosphoenolpyruvate to other phosphates. The obvious choice for the phosphoryl acceptor is ADP, as this is the biologically relevant nucleophile in most cases. A number of other nucleophiles can also accept the phosphoryl group from phosphoenolpyruvate, depending on the particular enzymes involved. These alternative nucleophiles include water, inorganic phosphate and nucleoside diphosphates, and are typically involved in the carboxylation of phosphoenolpyruvate to produce oxaloacetate **(Figure 3.8)**.

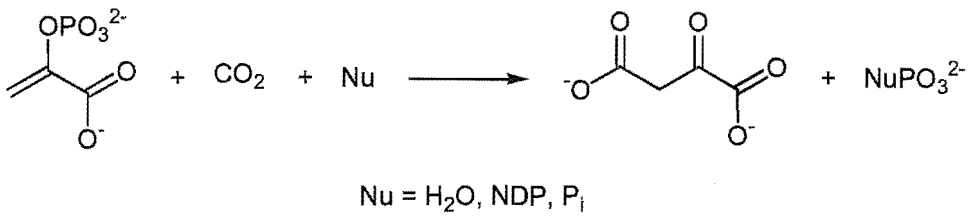


Figure 3.8. Carboxylation of phosphoenolpyruvate.

The enzymatic phosphorylation reactions of phosphoenolpyruvate are particularly interesting in light of the ability of Fe^{2+} to catalyse pyrophosphate formation from phosphoenolpyruvate and phosphate. All the enzyme-catalysed phosphorylations occur by direct attack of the nucleophile on the phosphoryl group of phosphoenolpyruvate, no phosphorylated enzyme intermediates are involved.[2] This is also the case in the Fe^{2+} catalysed reaction, where phosphoryl group transfer occurs directly between phosphoenolpyruvate and phosphate. The enzymatic reactions also have an absolute requirement for metal ions. Pyruvate kinase, which catalyses the reaction of phosphoenolpyruvate and ADP, requires two divalent metal ions, usually Mg^{2+}, as well as a monovalent metal ion such as K^{+}. The enzymes that catalyse the carboxylation of phosphoenolpyruvate, in conjunction with phosphoryl transfer, also require divalent metal ions.

The ability of Fe^{2+} to catalyse the phosphorylation of AMP and ADP using phosphoenolpyruvate as the phosphoryl donor was investigated in light of the similarities with the enzyme catalysed reactions. The reaction between phosphoenolpyruvate and either AMP or ADP was carried out in the presence of 0.1M Fe^{2+} at a pH of 6.5. These conditions were chosen as they had been determined previously to be optimal for the formation of pyrophosphate.

The loss of phosphoenolpyruvate in the presence of the different phosphoryl acceptors (phosphate, AMP and ADP) used in this study is shown below **(Figure 3.9)**.

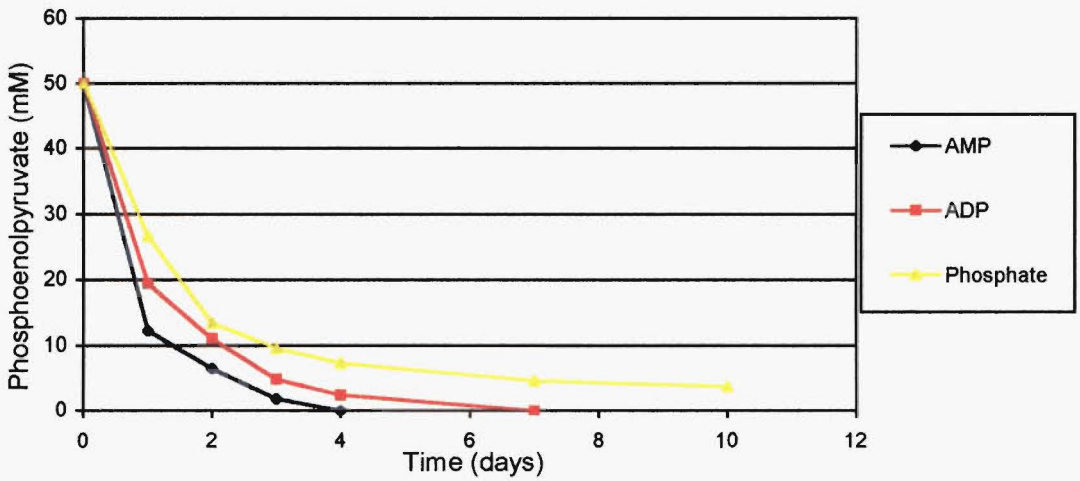


Figure 3.9. Rate of phosphoenolpyruvate loss in the presence of Fe(II) and different phosphoryl acceptors.

Buffered mixtures containing 0.05M phosphoryl acceptor (phosphate, AMP or ADP), 0.05M phosphoenolpyruvate and 0.1M ferrous sulfate were incubated at 45°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with potassium cyanide.

The rate of phosphoenolpyruvate loss was higher when AMP and ADP were used as the phosphoryl acceptors, compared with the rate of phosphoenolpyruvate loss when phosphate was the phosphoryl acceptor. A possible explanation for this is the following. Fe^{2+} is expected to form the strongest complexes with phosphate,

followed by Fe^{2+}-ADP complexes and then Fe^{2+}-AMP complexes. This means that the amount of Fe^{2+} not bound to the phosphoryl acceptors will be highest in the case of AMP and lowest in the case of phosphate. As we have already seen (see figure 3.5), the reactivity of phosphoenolpyruvate increases with increasing levels of Fe^{2+}. This may the case here, except that the amount of Fe^{2+} available to bind to the phosphoenolpyruvate is governed by the ability of the phosphoryl acceptor to bind Fe^{2+}.

Unfortunately phosphoryl transfer from phosphoenolpyruvate to AMP **(Figure 3.10)** or ADP **(Figure 3.11)** was not observed in either case. While this was disappointing, it was consistent with the finding in the previous chapter that phosphoryl transfer from acetyl phosphate to AMP or ADP was not observed.

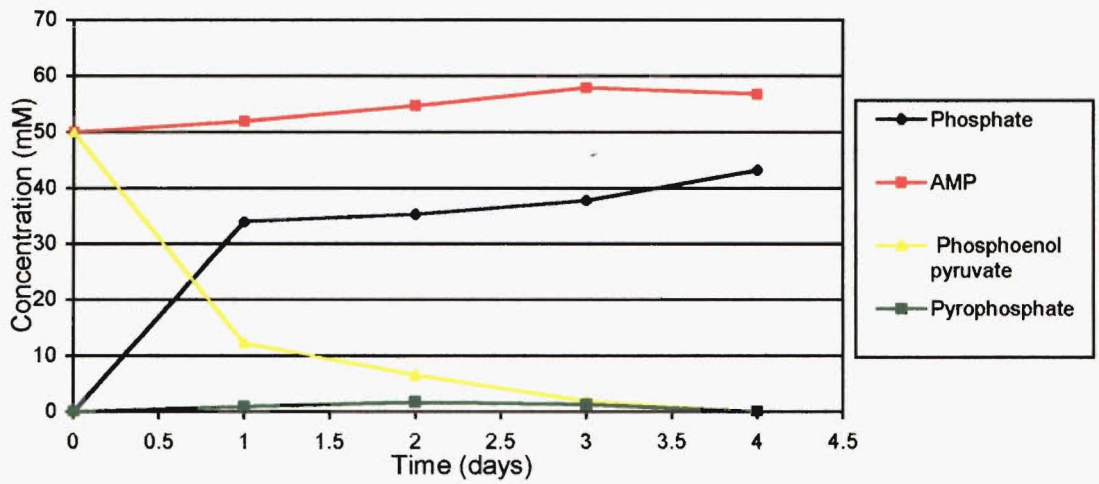


Figure 3.10. Attempted phosphoryl transfer from phosphoenolpyruvate to AMP.

Buffered mixtures containing 0.05M AMP, 0.05M phosphoenolpyruvate and 0.1M ferrous sulfate were incubated at 45°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with potassium cyanide.

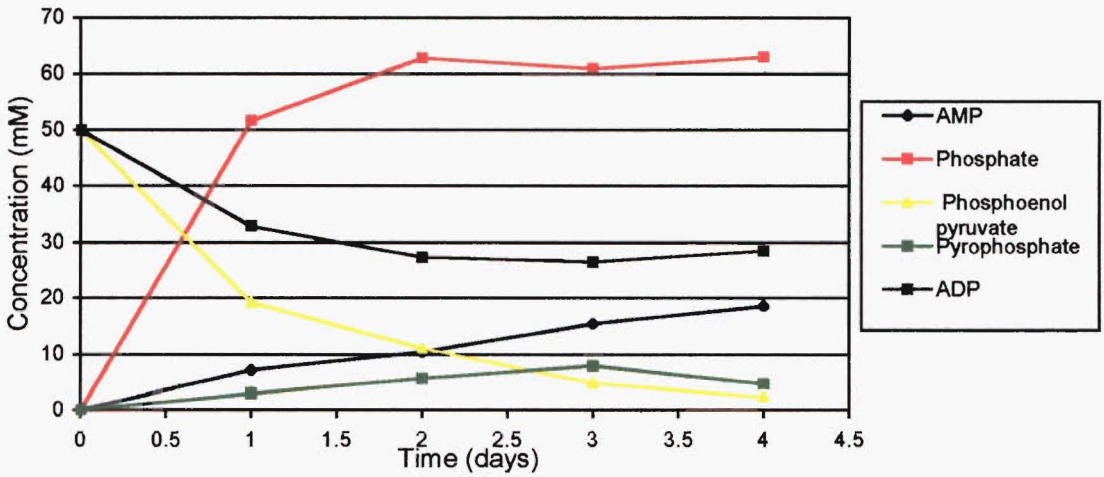


Figure 3.11. Attempted phosphoryl transfer from phosphoenolpyruvate to ADP.

Buffered mixtures containing 0.05M ADP, 0.05M phosphoenolpyruvate and 0.1M ferrous sulfate were incubated at 45°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with potassium cyanide.

The formation of pyrophosphate was observed both in the presence of AMP and in the presence of ADP. This presumably arises because the hydrolysis of phosphoenolpyruvate liberates inorganic phosphate, which can then act as a nucleophile. The yield of pyrophosphate was ~14% when ADP was present and ~2% in the presence of AMP. The greater yield of pyrophosphate in the presence of ADP, compared with AMP present, may be due to an increase in the amount of phosphate present as a result of the hydrolysis of ADP. Over the course of the reaction ~40% of the ADP hydrolysed, generating phosphate and AMP. Alternatively, ADP may have a positive effect on pyrophosphate formation. This is unlikely, since in the analogous reaction using acetyl phosphate as the phosphoryl donor, the formation of pyrophosphate was not observed.

Again, similar arguments to those used in the previous chapter may be invoked to explain the inability of Fe^{2+} to catalyse phosphoryl transfer nucleophiles other than inorganic phosphate. AMP and ADP may form chelates with Fe^{2+} and this may

prevent the co-precipitation of phosphoenolpyruvate and the nucleotide phosphate from occurring that is required for phosphoryl transfer to take place. In addition, precipitates formed from Fe^{2+} and organic phosphates are likely to be considerably different than precipitates consisting mainly of Fe^{2+} and inorganic phosphate. Since it appears that the ferrous phosphate precipitate is essential for phosphoryl transfer, changes in the precipitate will affect the phosphoryl transfer activity. It would be worthwhile investigating the precipitates that give rise to phosphoryl transfer.

3.3 Chemistry of 2-Phosphoglycerate and 3-Phosphoglycerate.

In glycolysis, the first ATP generating step is the phosphorylation of ADP by 1,3-diphosphoglycerate, an acyl phosphate. This also generates 3-phosphoglycerate, which is converted in two steps into phosphoenolpyruvate **(Figure 3.12)**. The first step involves the isomerisation of 3-phosphoglycerate to 2-phosphoglycerate, a transfer of the phosphoryl group from the 3-hydroxyl to the 2-hydroxyl. The next step is the dehydration of 2-phosphoglycerate to yield phosphoenolpyruvate. The purpose of the isomerisation step becomes clear; the phosphate ester is dehydrated to an enol, trapped in this form as the phosphoryl derivative so that it is unable to tautomerise to the more stable α-keto-acid. Interestingly, both of these reactions are thermodynamically favourable under physiological conditions, with a free energy change of –0.6 and –2.4 kJ/mol respectively, yet the product of these two reactions, phosphoenolpyruvate, is a high-energy phosphate that is used to phosphorylate ADP in the second ATP generating step of glycolysis.

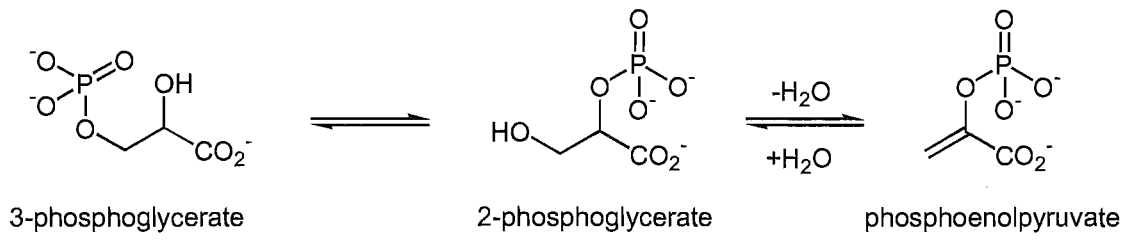


Figure 3.12. The formation of phosphoenolpyruvate from 3-phosphoglycerate.

The ability to generate phosphoenolpyruvate from either 3-phosphoglycerate or 2-phosphoglycerate is of interest in terms of understanding how phosphoenolpyruvate became incorporated into an emerging metabolism. This would provide a means of generating phosphoanhydride bonds from relatively stable precursors that may have been present on the early earth. Kolb and Orgel[5] have demonstrated that the phosphorylation of glycerate by trimetaphosphate produces both 2-phosphoglycerate and 3-phosphoglycerate **(Figure 3.13)**. Trimetaphosphate has been proposed as a prebiotic phosphorylating agent as it is produced during the hydrolysis of inorganic polyphosphates[6] and has also been produced under conditions that simulate volcanic activity.[7]

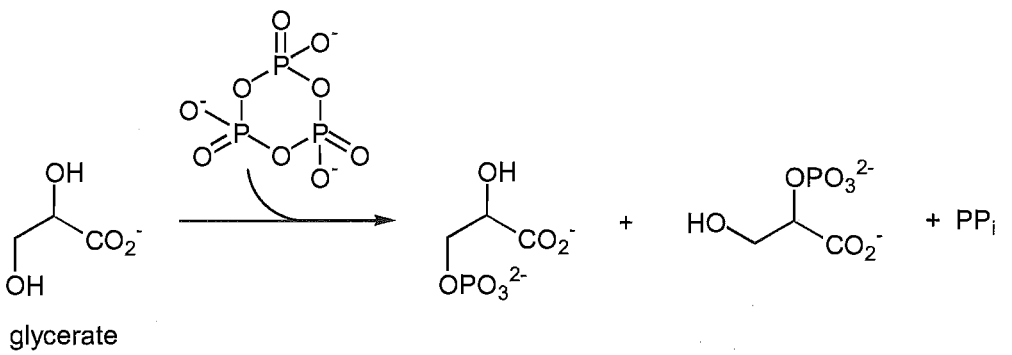


Figure 3.13. Phosphorylation of glycerate by trimetaphosphate.

3.3.1 The dehydration of 2-phosphoglycerate to phosphoenolpyruvate.

The dehydration of 2-phosphoglycerate is catalysed by the enzyme enolase and has an absolute requirement for a divalent metal ion, typically Mg^{2+}. The interconversion of 2-phosphoglycerate and phosphoenolpyruvate has an equilibrium constant of 6.7 in favour of phosphoenolpyruvate[2] **(Figure 3.14)**.

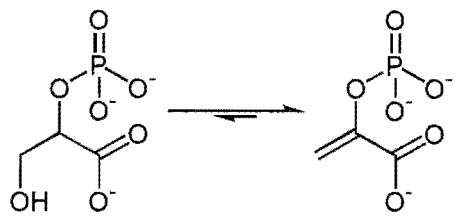


Figure 3.14. Dehydration of 2-phosphoglycerate.

This is unusual for a dehydration reaction, as intuitively the hydration reaction would be expected to be favoured in aqueous solution given the high concentration of water. The favourable equilibrium constant for dehydration to phosphoenolpyruvate raises the possibility that Fe^{2+} could catalyse the dehydration of 2-phosphoglycerate. This hypothesis was stimulated by the knowledge that a number of iron-dependent enzymes catalyse dehydration reactions. For example the enzyme aconitase, which catalyses the elimination of water from citrate and isocitrate, has an Fe_4S_4 cluster at its active site that the substrates bind to via one of the Fe ions **(Figure 3.15)**.

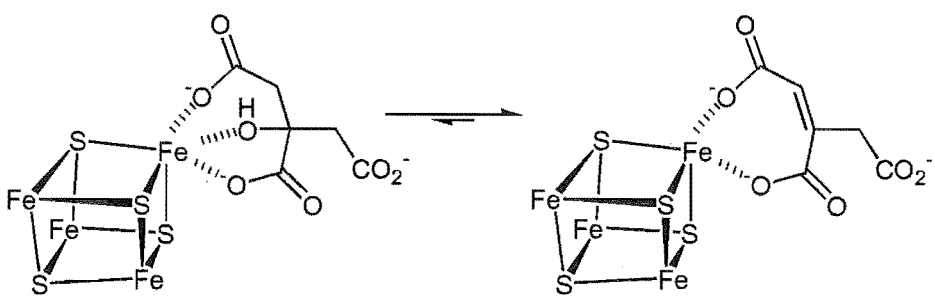


Figure 3.15. Dehydration of citrate by aconitase.

3.3.2 The isomerisation of 3-phosphoglycerate to 2-phosphoglycerate.

2-Phosphoglycerate is formed through the isomerisation of 3-phosphoglycerate, catalysed by the enzyme phosphoglycerate mutase. In some cases this is achieved via a 1,2 intramolecular phosphoryl transfer[8] **(Figure 3.16)**, but in other cases the reaction proceeds via 2,3-bisphosphoglycerate[9], in which case the enzyme contains a phosphohistidine residue at the active site **(Figure 3.17)**. Whether or not the intramolecular reaction occurs via a direct transfer, or an enzyme bound intermediate is involved, is unclear.

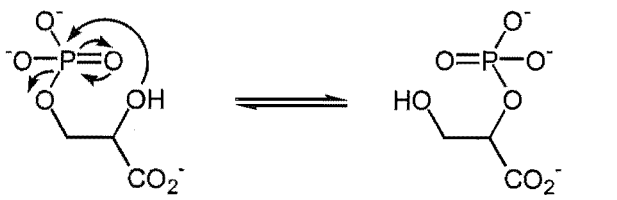


Figure 3.16. 1,2-Intramolecular phosphoryl transfer.

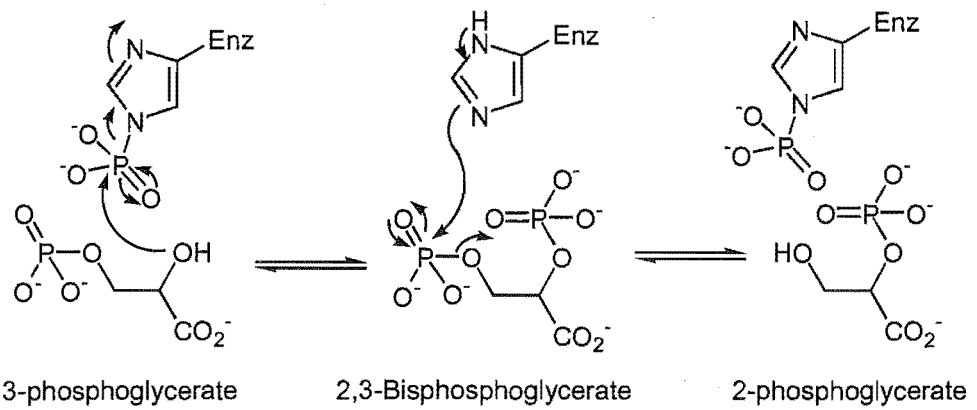


Figure 3.17. Intermolecular phosphoryl transfer.

In light of the known ability of Fe^{2+} to catalyse both phosphoryl transfer and dehydration reactions, the chemistry of 2-phosphoglycerate and 3-phosphoglycerate in the presence of ferrous ions was investigated.

The hydrolysis of 2-phosphoglycerate and 3-phosphoglycerate in the presence of Fe^{2+} was monitored at 95°C and at a pH of 6.5. The hydrolysis of another phosphate ester, AMP, was also monitored under the same conditions for comparative purposes **(Figure 3.18)**.

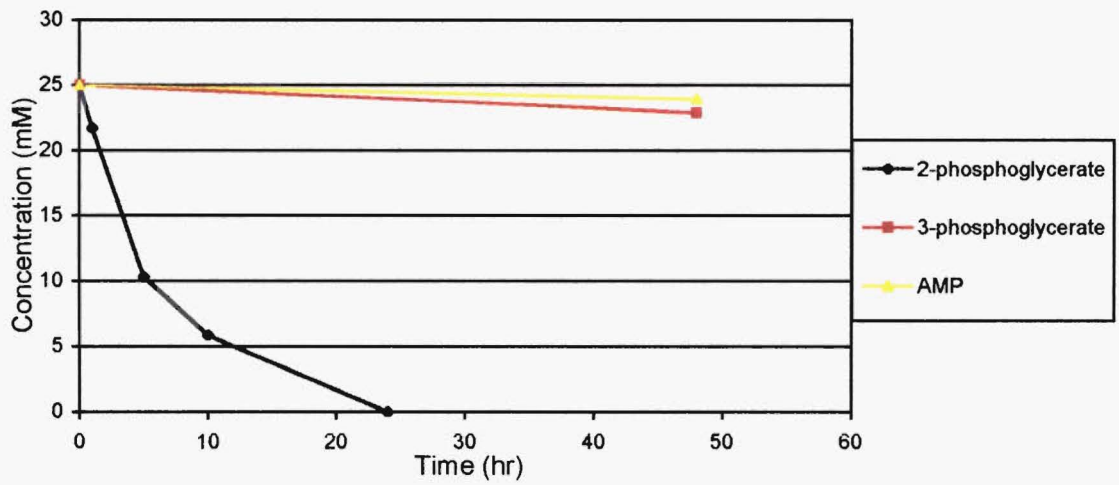


Figure 3.18. Hydrolysis of phosphate esters in the presence of Fe(II).

Buffered mixtures containing 0.025M phosphate ester and 0.1M ferrous sulfate were incubated at 95°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with potassium cyanide.

Compared to the hydrolysis of the other phosphate esters, 3-phosphoglycerate and AMP, the hydrolysis of 2-phosphoglycerate is relatively fast. The hydrolysis of 2-phosphoglycerate was complete within one day, while under the same conditions, 3-phosphoglycerate and AMP required many days to hydrolyse. The higher rate of hydrolysis of 2-phosphoglycerate may be due to two factors: 2-phosphoglycerate may be intrinsically more reactive than other phosphate esters; or, 2-phosphoglycerate may hydrolyse via an alternative pathway that is not available to the other phosphate esters.

The reactivity of 2-phosphoglycerate may be due to the participation of the carboxyl group during hydrolysis. For instance, intramolecular transfer of the phosphoryl

group to the carboxylate would result in the formation of an acyl phosphate, which would be expected to hydrolyse very rapidly **(Figure 3.19)**. In 2-phosphoglycerate, such an intramolecular transfer may be favourable as the transition state would be a five-membered ring. However, this mechanism might also be expected to occur in the hydrolysis of 3-phosphoglycerate, in which case the transition state for phosphoryl transfer would be a six-membered ring. The formation of five-membered and six-membered rings in the transition state are both conducive to intramolecular transfers of this type.

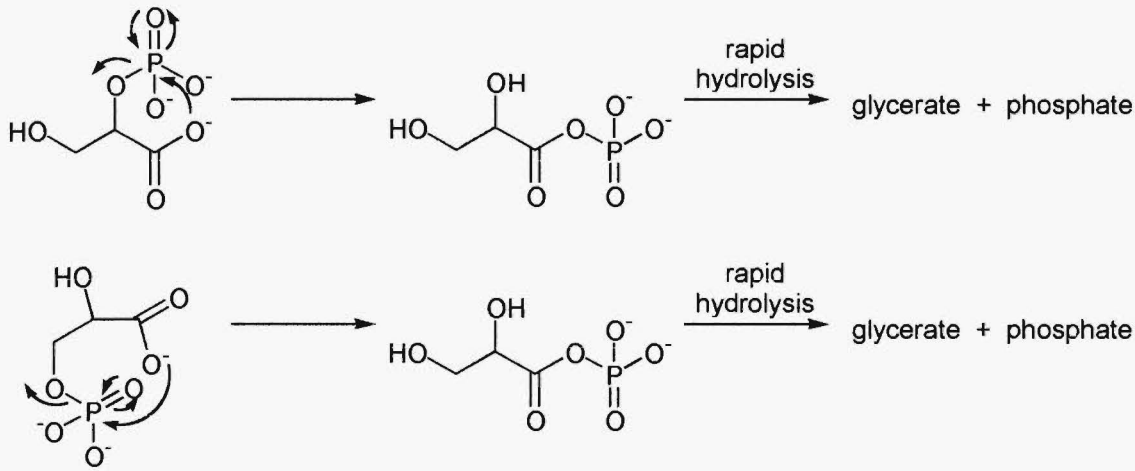


Figure 3.19. Hydrolysis of 2-phosphoglycerate and 3-phosphoglycerate with participation of the carboxyl group.

Another possible reason may be that unfavourable electrostatic interactions between the phosphate and the carboxylate are greater in 2-phosphoglycerate than they are in 3-phosphoglycerate, resulting in some strain in the molecule **(Figure 3.20)**.

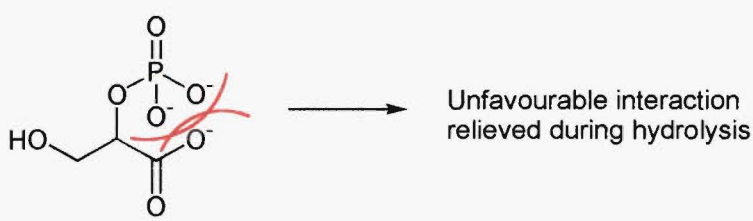


Figure 3.20. Possible electrostatic interaction in 2-phosphoglycerate.

Alternatively, the hydrolysis of 2-phosphoglycerate may proceed via dehydration to phosphoenolpyruvate, which would be expected to hydrolyse very rapidly. The favourable equilibrium constant for dehydration to phosphoenolpyruvate shows that there is no thermodynamic barrier to this process, although the dehydration may be very slow. The formation of pyruvate would be conclusive evidence that hydrolysis proceeds via phosphoenolpyruvate, since the formation of pyruvate can only come about through the hydrolysis of phosphoenolpyruvate. The products of the alternative hydrolysis are glycerate and phosphate **(Figure 3.21)**.

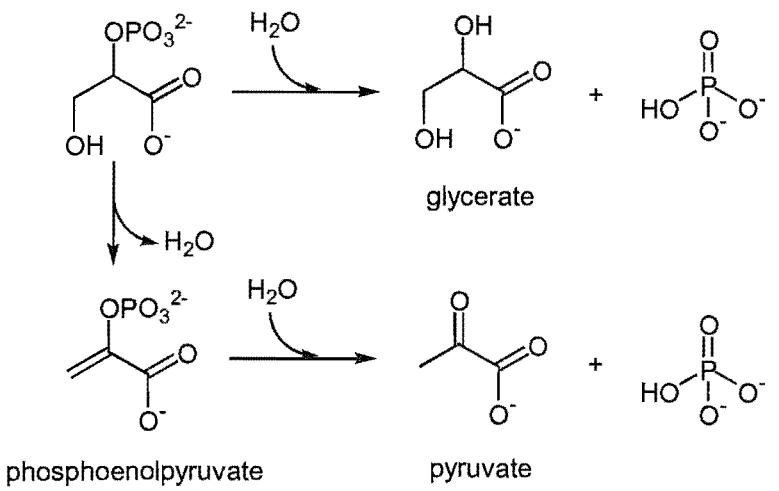


Figure 3.21. Two pathways for the hydrolysis of 2-phosphoglycerate.

A preliminary investigation was carried out after the hydrolysis was complete to test for the presence of pyruvate. TLC analysis, using iodine as a visualising agent, indicated that a compound in the reaction co-eluted with an authentic sample of pyruvate. A component of the reaction mixture also reacted with 2,4-DNP, which reacts with carbonyl groups to form yellow hydrazones. Although this was evidence for the presence of pyruvate, further proof was required to confirm that pyruvate was present.

A common method for the identification of volatile organic compounds is GC-MS analysis, which could unambiguously verify the presence of pyruvate. There are two problems with GC-MS of pyruvate: it's lack of volatility and the ease with which it

undergoes decarboxylation. The decarboxylation of pyruvate generates carbon dioxide and a CH_3CO fragment, which is difficult to detect because it has a small molecular mass, similar to that of solvent molecules. Both of these problems can be overcome by esterification of the carboxylate group **(Figure 3.20)**.

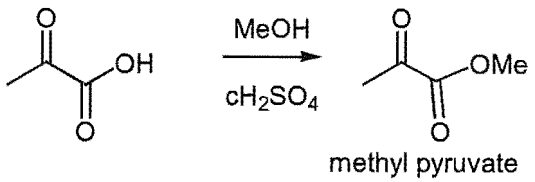


Figure 3.20. Methylation of pyruvic acid.

Two attempts were made to esterify any pyruvate in the reaction mixture, both using methanol and concentrated sulfuric acid. Firstly, the reaction mixture was acidified and extracted with ether, and the residue remaining after evaporation of the ether was methylated. An attempt was also made to methylate any pyruvate remaining in the reaction mixture directly. However GC-MS analysis of the methylated samples failed to detect any methyl pyruvate in either sample. This may have been due to the absence of pyruvate in the reaction mixture, or to a failure to methylate any pyruvate.

Because of the difficulties associated with GC-MS analysis of pyruvate, another method was required, preferably one that did not require any modification of pyruvate prior to the analysis. An enzyme assay, using L-lactate dehydrogenase, seemed as if it would be suitable. In the presence of NADH, this enzyme reduces pyruvate to L-lactate, concomitantly producing NAD^+, the oxidised form of NADH **(Figure 3.21)**. The reaction can be easily monitored by measuring the absorbance of the solution at 340nm, since at this wavelength NADH absorbs strongly while NAD^+ does not absorb at all.

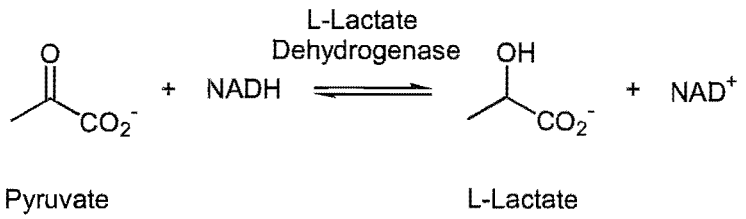


Figure 3.21. Reduction of pyruvate to L-lactate by NADH.

The hydrolysis of 2-phosphoglycerate in the presence of Fe^{2+} was repeated and tested for pyruvate using the lactate dehydrogenase assay. However, no pyruvate was detected in aliquots of the reaction mixture, indicating that the hydrolysis of 2-phosphoglycerate does not proceed via the intermediacy of phosphoenolpyruvate.

3.4 Chapter Summary.

This chapter has demonstrated that Fe(II) is an efficient catalyst for the formation of pyrophosphate from phosphoenolpyruvate and phosphate. The reaction was found to be pH dependent, with a maximum yield occurring at pH 6.5. This may be due to an increase in the binding of phosphate and phosphoenolpyruvate to Fe^{2+} as the pH is raised. The decrease in the yield of pyrophosphate above pH 7 may be due to the formation of insoluble iron hydroxides.

Pyrophosphate formation was demonstrated to be dependent on the amount of Fe^{2+} present. This is most likely due to the selective precipitation of phosphate when the amount of Fe^{2+} present is limiting. Co-precipitation of phosphoenolpyruvate and phosphate, which is required for the formation of pyrophosphate, only occur when the amount of Fe^{2+} available is not limiting.

Phosphoryl transfer from phosphoenolpyruvate to AMP and ADP was not observed under the conditions used. This may be due to changes in the type of precipitate that formed in the presence of the organic phosphates. In both cases, the formation of

pyrophosphate was observed as inorganic phosphate is released during the hydrolysis of phosphoenolpyruvate.

The hydrolysis of 2-phosphoglycerate, 3-phosphoglycerate and AMP in the presence of Fe^{2+} was also examined. 2-Phosphoglycerate hydrolysed rapidly in the presence of Fe^{2+}, in contrast to 3-phosphoglycerate and AMP, which were much more stable. The hydrolysis of 2-phosphoglycerate did not proceed via the intermediacy of phosphoenolpyruvate as no pyruvate was detected in the reaction. The reason for the lability of 2-phosphoglycerate, compared with other phosphate esters, is not clear at this stage.

3.5 References for Chapter Three.

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4 Polyphosphate Chemistry.

4.1 Introduction.

In the previous two chapters, the ability of Fe^{2+} to catalyse the formation of pyrophosphate, from inorganic phosphate and an activated phosphoryl donor, has been demonstrated. The formation of pyrophosphate in the presence of Fe^{2+} may have played an important role in the origin of metabolism. Pyrophosphate has been put forward as a precursor of ATP, as it could have been a source of readily utilisable energy for an emerging metabolism.[1] While the ability to form pyrophosphate under conditions that may have been similar to those on the early earth is very interesting, it would be of no use if pyrophosphate did not undergo further chemistry. Obviously, if pyrophosphate is to be important in the evolution of metabolism, the free energy that it contains must be made available to an emerging metabolism. For this reason, the ability of pyrophosphate and other phosphoanhydrides to undergo phosphoryl transfer reactions was investigated **(Figure 4.1)**.

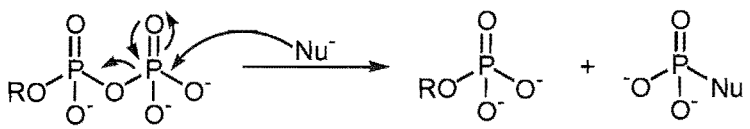


Figure 4.1. Phosphoryl transfer from a phosphoanhydride.

This chapter describes investigations into the reactions of various phosphoanhydrides in the presence of Fe^{2+}. The hydrolysis of inorganic polyphosphates, pyrophosphate and tripolyphosphate, both in the presence and in the absence of Fe^{2+} is described first. The effect of amines on the hydrolysis of pyrophosphate is also described. Although hydrolysis of a phosphoanhydride bond results in loss of the free energy that it contains, it is the easiest phosphoryl transfer reaction to study. The results from the hydrolysis of the inorganic polyphosphates are used to understand the reactions of ATP and ADP, two

biologically important polyphosphates, in the presence of Fe^{2+}. Attempts to catalyse phosphoryl transfer from pyrophosphate to nucleophiles other than water are also described.

4.2 Metal Ion Coordination with Polyphosphates.

Any attempt to rationalise the hydrolysis chemistry of polyphosphates in the presence of Fe^{2+} requires an understanding of the coordination chemistry of polyphosphates with Fe^{2+}. There is a paucity of data in the literature regarding the coordination of Fe^{2+} with phosphate ligands. Unfortunately the system that is being studied in this thesis, namely precipitates containing Fe^{2+} and phosphates, does not directly provide any information about the coordination involved. However there is a large amount of data available regarding the coordination chemistry of phosphates with other divalent metal ions and this provides some insight into the coordination chemistry that may be involved in this study. The coordination chemistry of Fe(II) with polyphosphates would be expected to be similar to that observed for other divalent transition metal ions.

4.2.1 Pyrophosphate and tripolyphosphate.

The symmetrical nature of pyrophosphate and tripolyphosphate means that the types of complexes that these two ligands can form with divalent metal ions are limited. Complexes have been isolated and characterised with pyrophosphate coordinating to the metal centre in a unidentate and a bidentate fashion[2] **(Figure 4.2)**.

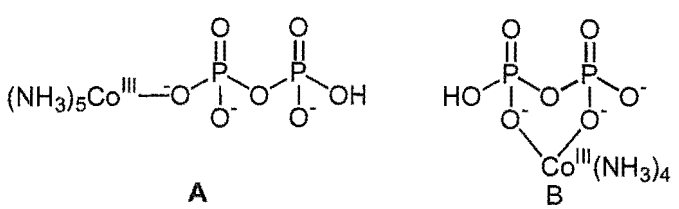


Figure 4.2. Complexes showing unidentate (A) and bidentate (B) coordination.

However in solution it is most likely that coordination of pyrophosphate to a metal ion involves both phosphates, forming a chelate **(Figure 4.3)**.

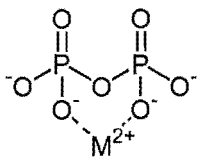


Figure 4.3.

Complexes containing the α,γ-bidentate and the α,β,γ-tridentate tripolyphosphate ligands have also been characterised[3,4] **(Figure 4.4)**, but again, in solution α,β-bidentate coordination will probably predominate because of the chelate effect **(Figure 4.5 (A))**. In the presence of excess metal ions, more than one metal ion may coordinate to tripolyphosphate, with the second metal ion coordinating to the γ-phosphate **(Figure 4.5 (B))**.

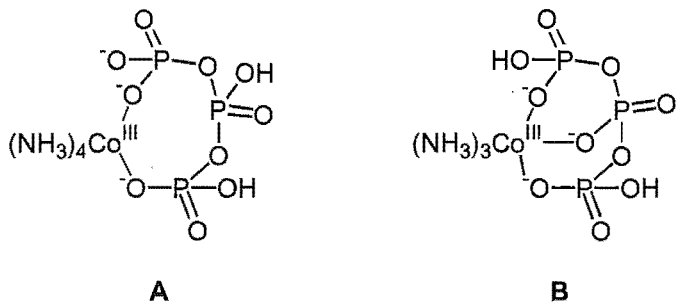


Figure 4.4. Bidentate (A) and tridentate (B) coordination of tripolyphosphate.

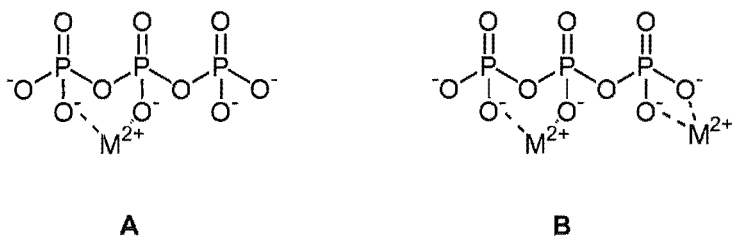


Figure 4.5. Possible coordination of divalent metal ions to tripolyphosphate.

4.2.2 ADP and ATP.

The coordination chemistry of ADP and ATP is rather more complicated than the coordination chemistry of their analogues, pyrophosphate and tripolyphosphate. This is because the presence of the adenosine group removes the symmetry found in the inorganic polyphosphates, and the adenosine group has a number of O-, and N-, donor atoms that can also coordinate to metal ions. The sites on the adenosine that could potentially coordinate metal ions include the ring nitrogens, N(1), N(3) and N(7), and the oxygens of the ribose, the hydroxyls and the ring oxygen **(Figure 4.6)**.

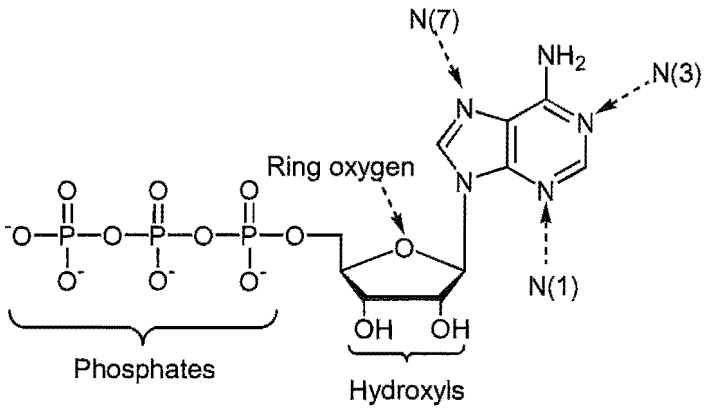


Figure 4.6. Potential coordination sites of ATP.

The primary amino group does not coordinate with metal ions as the pair of electrons on the nitrogen is delocalised within the aromatic system of the purine. Of these potential metal ion binding sites, N(7) is the most important because in the preferred *anti* conformation of ATP the N(7) faces towards the phosphates **(Figure 4.7)**.

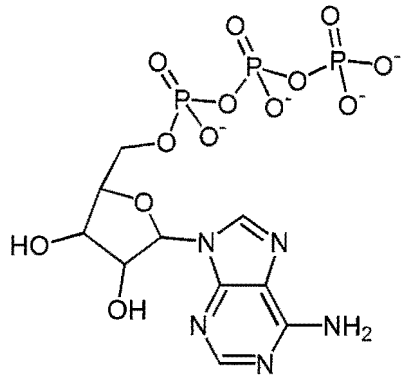


Figure 4.7. *Anti*-configuration of ATP.

Metal ions coordinate primarily to the phosphates of ADP and ATP, except when the metal ion is capable of co-ordinating with nitrogen. If this is the case, then a macrochelate involving metal ion coordination of N(7) of the adenine ring and the β- and γ- phosphates may also form. In the macrochelate, the α-phosphate does not interact directly with the metal ion due to steric constraints. In solution, an equilibrium between the open complex and the macrochelate exists, with the extent of macrochelate formation depending on the particular metal ion present[5] **(Figure 4.8)**.

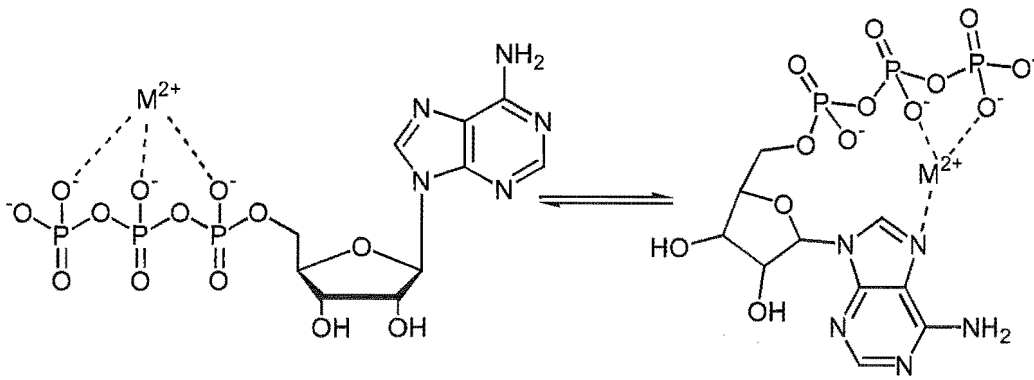


Figure 4.8. Open and macrochelate complexes of M^{2+}-ATP.

ADP forms macrochelates with divalent metal ions to a greater extent than ATP does. For example, about 80% of the Zn^{2+}-ADP complex in solution exists as the macrochelate, while for the Zn^{2+}-ATP complex about 30% of the complex is the

macrochelate. The extra phosphate present in ATP may favour the open complex with the metal ion bound only to the phosphates.[5]

4.2.3 Biological reactions of ATP?

In biological systems, ADP and ATP are invariably present as the Mg^{2+} complexes because of the high relative concentration of Mg^{2+} compared to other divalent metal ions. Mg^{2+} displays a strong preference for oxygen-donor ligands, and as a result, interacts exclusively with the phosphoryl groups of ADP and ATP. In the case of ADP, Mg^{2+} coordinates with both α- and β- phosphates, while for ATP, NMR evidence suggests that Mg^{2+} binds to the β,γ-phosphates and may also coordinate with the α-phosphate[6] **(Figure 4.9)**.

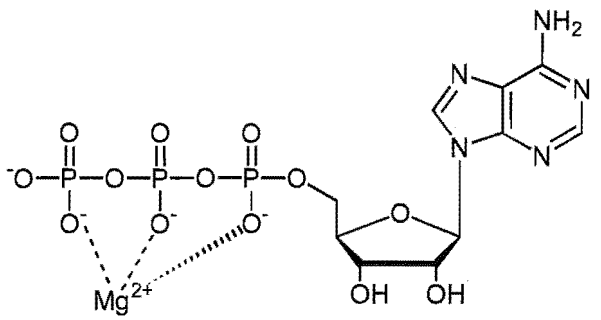


Figure 4.9. Mg^{2+}-ATP complex.

Although both ADP and ATP could potentially serve as the source of energy for many biological transformations, it is invariably ATP that is used. Almost all of the reactions requiring ADP are involved in the regeneration of ATP. The predominant use of ATP may be because of the extra versatility in group transfer chemistry that the γ-phosphate provides. ATP can undergo a variety of phosphoryl transfer reaction depending on which phosphorus centre is the subject of nucleophilic attack. The two most common reactions are nucleophilic attack at the γ-phosphorus, leading to phosphoryl transfer; and attack at the α-phosphorus, which gives adenylyl transfer. Nucleophilic attack can also occur at the β-phosphorus, resulting in pyrophosphoryl transfer, or at the C-5′ of the ribose unit, which transfers an adenosyl unit **(Figure**

4.10). Examples of all these types of reactions can be found in nature, although pyrophosphoryl and adenosyl transfer are quite rare.

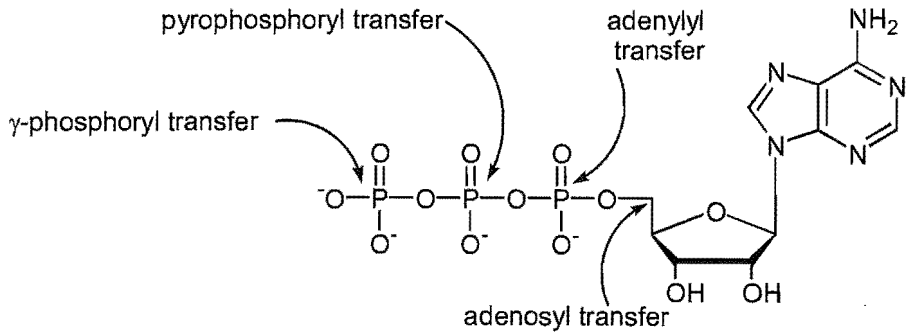


Figure 4.10. The different sites of nucleophilic attack on ATP.

Transfer of the γ-phosphoryl group is most common and is used to drive most thermodynamically unfavourable processes. Many enzymes and metabolic intermediates are also phosphorylated using the γ-phosphate of ATP. Nucleotidyl transfer is often used to drive biosynthetic reactions. This mechanism of NTP cleavage is used in the biosynthesis of peptides and polysaccharides. Nucleotidyl transfer is discussed in more detail below. Pyrophosphoryl transfer is very rare in biology, and only a few examples are known. These include the formation of thiamine pyrophosphate, an important coenzyme, and phosphoribosyl pyrophosphate, which is involved in the biosynthesis of nucleotides. The only known examples of adenosyl transfer are found in the biosyntheses of *S*-adenosyl methionine and of vitamin B_{12} **(Figure 4.11)**.[7]

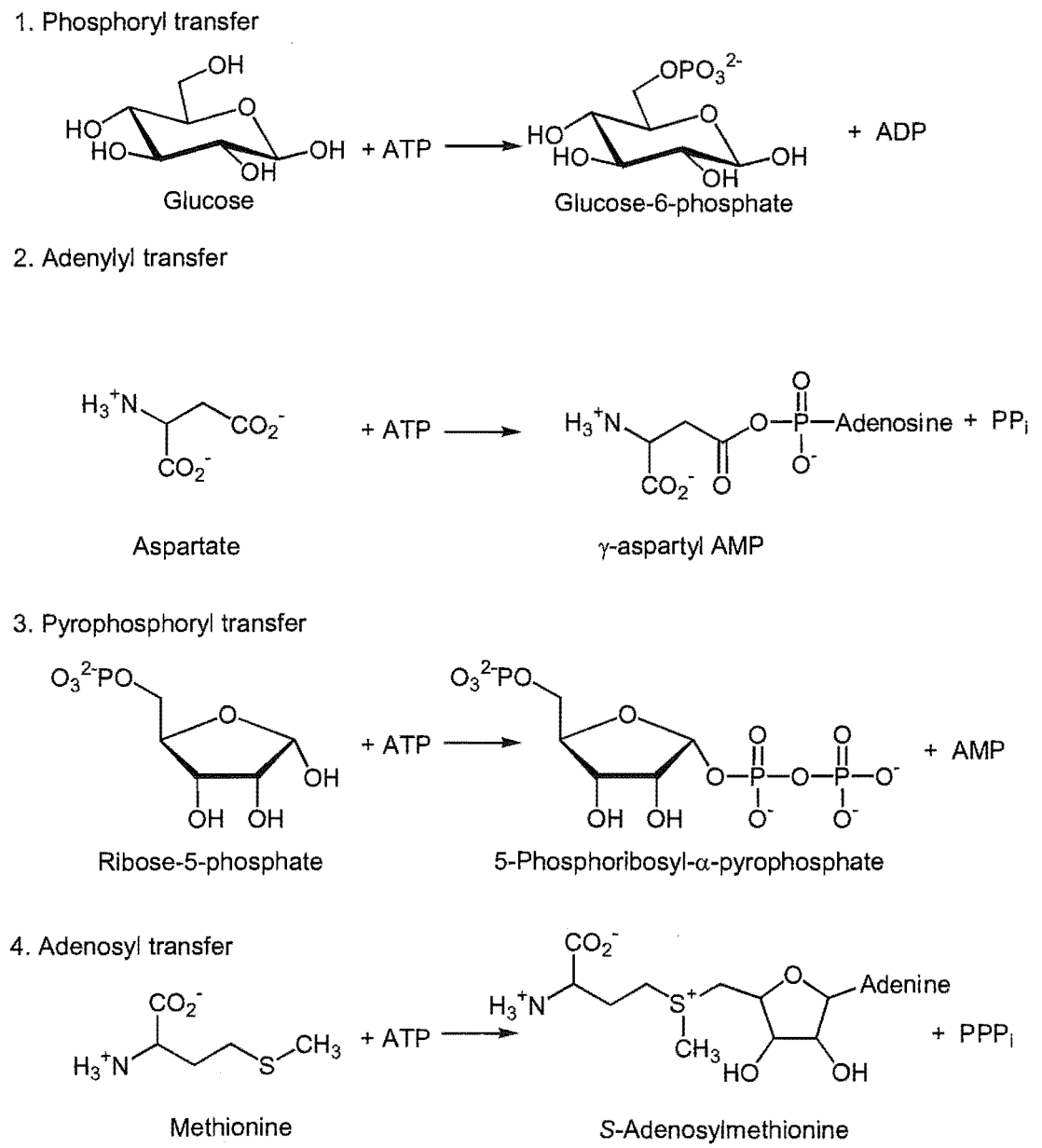
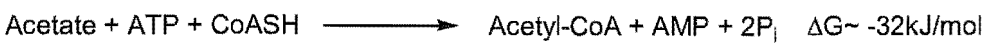


Figure 4.11. Group transfer reactions of ATP

Nucleotidyl transfer is often used to ensure that a biosynthetic reaction is made irreversible. Irreversibility is achieved by the hydrolysis of the pyrophosphate produced. For example, the two-enzyme system (acetate kinase and phosphotransacetylase) used to convert acetate to the activated acetyl-CoA, via the intermediate acetyl-AMP, has an overall equilibrium constant of ~1. This is because the energy of the phosphoanhydride bond of ATP is conserved in the energy of the thioester bond. However, hydrolysis of

the pyrophosphate produced liberates an extra 32kJ/mole and makes the equilibrium constant for the reaction closer to 10^4.



The hydrolysis of pyrophosphate is catalysed by the enzyme inorganic pyrophosphatase, a very active enzyme found in most organisms. Perhaps not surprisingly, inorganic pyrophosphatase requires a number of divalent metal ions, usually Mg^{2+}, although Mn^{2+}, Zn^{2+} and Co^{2+} are also active.[8] Inorganic pyrophosphatase serves to keep the intracellular concentration of pyrophosphate very low. This is why biosynthetic reactions often utilise nucleotidyl transfer reactions, since irreversibility can be achieved by hydrolysis of pyrophosphate that is liberated.

While ADP does not substitute for ATP in reactions requiring a source of free energy, in some organisms pyrophosphate is used as a source of free energy. Enzymes that have been found to utilise pyrophosphate include a pyrophosphate: fructose 6-phosphate 1-phosphotransferase as well as an acetate kinase.[9] Some bacteria have been shown to synthesise pyrophosphate via electron transport coupled phosphorylation, analogous to the synthesis of ATP via oxidative phosphorylation. [9] In addition, all cells contain some inorganic polyphosphates, large polymers of phosphate, which can be used as a phosphoryl donor.[10] The current widespread use of inorganic polyphosphates supports the hypothesis that these may have been precursors to ATP as a source of readily available free energy.

4.3 Hydrolysis of Pyrophosphate and Tripolyphosphate.

The hydrolysis of pyrophosphate and tripolyphosphate was investigated first, as these reactions are relatively uncomplicated. Knowledge of the factors effecting hydrolysis of pyrophosphate and tripolyphosphate will be useful in understanding the hydrolysis of ADP and ATP.

The standard free energy of hydrolysis of a phosphoanhydride bond is ~ -31kJ/mole, which represents an equilibrium constant of about 10^4 in favour of hydrolysis. Although pyrophosphate and tripolyphosphate are thermodynamically unstable, they are kinetically stable at neutral pH in aqueous solution. At neutral pH pyrophosphate exists predominantly as the trianion ($pK_{a(4)}$=8.2), while tripolyphosphate exists as the tetraanion ($pK_{a(5)}$=8.5).[11] Electrostatic repulsion accounts for the slow reaction of pyrophosphate and tripolyphosphate with nucleophiles.

4.3.1 The effect of metal ions on polyphosphate hydrolysis.

The stability of pyrophosphate and tripolyphosphate in the presence of metal ions was examined. Metal ions are often catalysts for the hydrolysis of phosphate compounds, as has been discussed previously. They can act as catalysts by withdrawing electron density from the phosphoryl group. In addition, metal ions can bind and activate water molecules, increasing the local concentration of hydroxide anions. This is an important role, especially when the pH of a solution is close to neutral and the concentration of hydroxide is very low.

The hydrolysis of pyrophosphate in the presence of a number of different metal ions has previously been investigated. Watanabe *et.al.*[12] studied the effect of a wide range of monovalent and divalent metal ions on the rate of pyrophosphate hydrolysis in the pH range 1-5. Li^+, Na^+, K^+, Ca^{2+} and Mg^{2+} were found to increase the rate of hydrolysis of pyrophosphate, while Mn^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} all inhibited pyrophosphate hydrolysis somewhat. Earlier, Rainey and co-workers[13] had found that various metal ions including Mg^{2+}, Ca^{2+}, Mn^{2+} and Co^{2+} had no significant effect on the rate of hydrolysis, but Cr^{3+} strongly inhibited hydrolysis. They had postulated that the effect of Cr^{3+} might be due to a different mode of chelating the pyrophosphate as described earlier. However, these results were all obtained under quite acidic conditions where precipitates do not form and may not be comparable to the results obtained here. Hofstetter and Martell investigated the hydrolysis of pyrophosphate at pH 6.29.[14] They found that divalent metal ions had no discernible effect on the rate, but metal ions of a higher oxidation state, such as Zr(IV), increased the rate of hydrolysis significantly.

The hydrolysis of pyrophosphate was investigated in the presence of the metal ions Fe^{2+}, Ni^{2+} and Zn^{2+}. The effect of Fe^{2+} on the hydrolysis of tripolyphosphate was also studied and compared to the hydrolysis of pyrophosphate. Previous studies had indicated that pyrophosphate was stable for long periods of time at temperatures below about 50°C. For this reason, higher temperatures were used to investigate the hydrolysis of pyrophosphate. The hydrolysis of pyrophosphate was carried out at 95°C in the presence of Fe^{2+}, Ni^{2+}, Zn^{2+} and FeS, and in the absence of any added metal ions as a control **(Figure 4.12.)**.

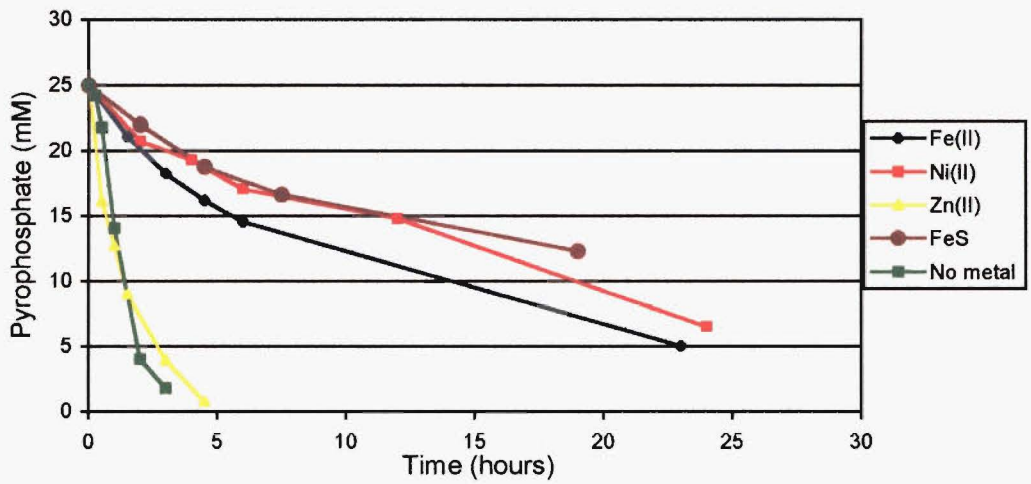


Fig. 4.12. The effect of metal ions on pyrophosphate hydrolysis.

Buffered mixtures containing 0.025M pyrophosphate and 0.1M Fe^{2+}, Ni^{2+}, Zn^{2+} or FeS were incubated at 95°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with potassium cyanide.

As can be seen from figure 4.12, the effect of metal ions on the hydrolysis of pyrophosphate is dependent on the metal ion present. In the presence of Zn^{2+}, the rate of pyrophosphate hydrolysis was identical to the rate of hydrolysis in the absence of added metal ions. By contrast, both Fe^{2+} and Ni^{2+} inhibited the hydrolysis of pyrophosphate, with Ni^{2+} inhibiting pyrophosphate hydrolysis to the greatest extent. Interestingly FeS had a very similar effect to Ni^{2+} on the rate of pyrophosphate hydrolysis.

The inhibition of pyrophosphate hydrolysis in the presence of Fe^{2+} and Ni^{2+} is probably due to both the chelation of pyrophosphate to the metal ion and the formation of a metal ion-pyrophosphate precipitate. In all the hydrolysis reactions involving metal ions a precipitate formed. Presumably this precipitate initially consisted of pyrophosphate and the metal ion, with the pyrophosphate being slowly replaced by phosphate as the hydrolysis proceeded. By sequestering the pyrophosphate within the precipitate, the precipitate may protect pyrophosphate from the solvent. Additionally, pyrophosphate will most likely coordinate to the metal ion via both phosphates, forming a six-membered chelate, which could stabilise the pyrophosphate with respect to hydrolysis.

The apparent lack of effect of Zn^{2+} may be due to competing effects. Zn^{2+} is generally a good catalyst for hydrolytic reactions, so in this case the net effect of catalysis relative to inhibition, due to precipitation and chelation, may be very small.

The formation of a six-membered chelate could also explain the effect of Fe^{2+} on the hydrolysis of tripolyphosphate. In the presence of Fe^{2+}, the hydrolysis of tripolyphosphate is accelerated relative to the hydrolysis of tripolyphosphate in the absence of Fe^{2+} **(Figure 4.13.)**.

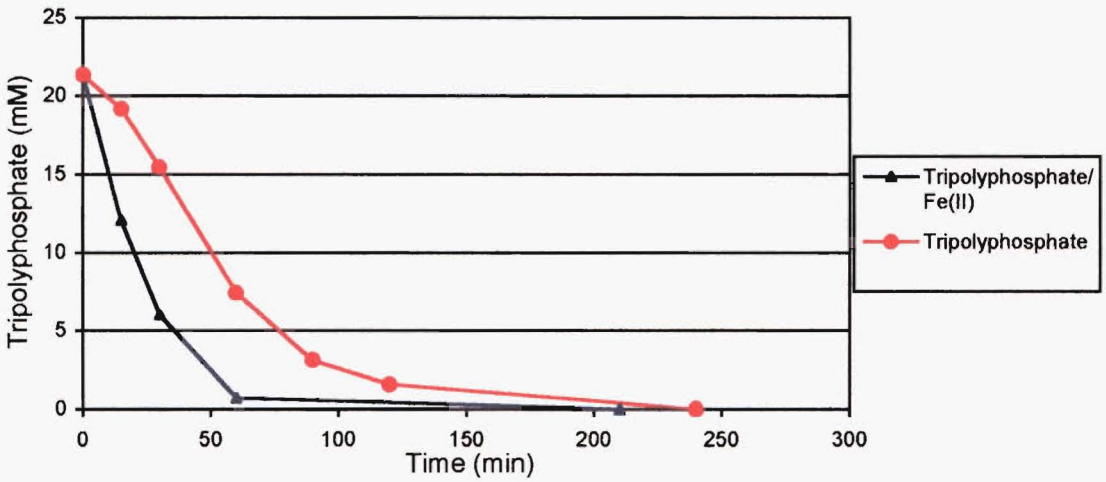


Fig. 4.13. The effect of Fe^{2+} on the hydrolysis of tripolyphosphate.

Buffered reactions containing 0.025M tripolyphosphate were incubated at 95°C with or without 0.1M Fe^{2+} present. Aliquots were removed and analysed by ^{31}P NMR after treatment with EDTA or potassium cyanide.

Chelation of the α- and β- phosphates to Fe^{2+} leaves the γ-phosphate free **(Figure 4.14 (A))**. Since an excess of Fe^{2+} compared to tripolyphosphate was used, the γ-phosphate is likely to be coordinated to a second Fe^{2+} ion **(Figure 4.14 (B))**.

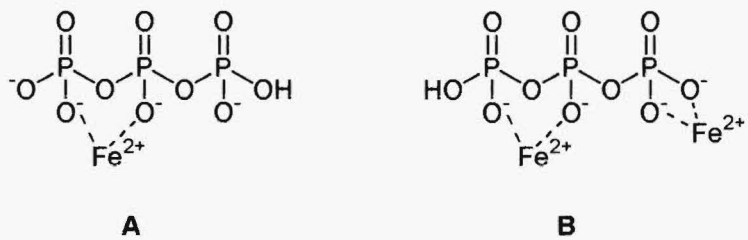


Figure 4.14. Coordination of Fe^{2+} to tripolyphosphate.

Chelation of a second metal ion would make tripolyphosphate susceptible to hydrolysis between the β- and γ- phosphates. The nucleophile may even be a metal bound hydroxide **(Figure 4.15)**. The Fe(II)-pyrophosphate group would also be a better leaving group than pyrophosphate.

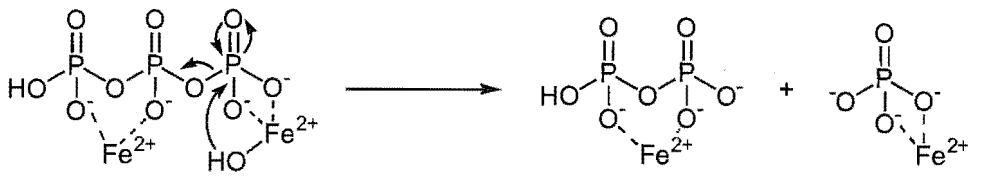


Figure 4.15. Hydrolysis of tripolyphosphate catalysed by two Fe^{2+} ions.

4.3.2 Effect of amines on the hydrolysis of pyrophosphate.

Amines are potentially good nucleophilic catalysts as they are effective nucleophiles when they are neutral. Enzymes that are involved in phosphoryl transfer often use amines as nucleophilic catalysts and a number of *N*-phosphorylated enzymes have been isolated. Glycine, imidazole and AMP were the amines used in this study, since these are biologically important examples **(Figure 4.16)**. Since this work was carried out at pH 6.5, the amines will be protonated to some extent. Glycine has a pK_a of 9.6, so it will be almost completely protonated[15]. Imidazole has a pK_a of 7.2, so a significant amount of imidazole will remain unprotonated,[15] while the N(7) of AMP has a pK_a of 4 and will be almost completely unprotonated.[6]

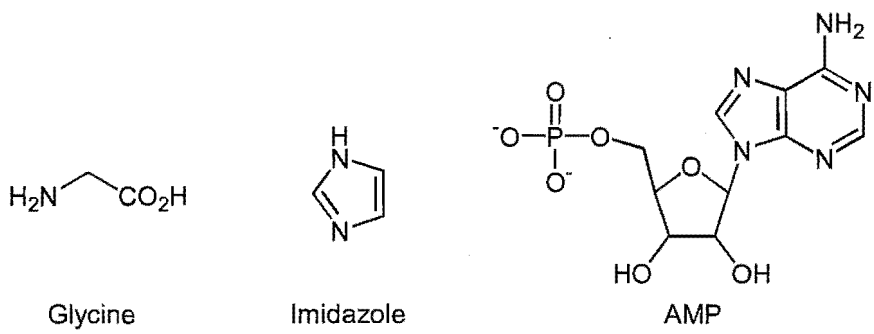


Figure 4.16.

Very few studies have been carried out on the influence of amines on the hydrolysis of pyrophosphate. Dimethylaniline is reported to have no effect on the rate of hydrolysis of pyrophosphate.[16] A few of the polyamine macrocycles extensively studied by Lehn and co-workers were found to catalyse pyrophosphate hydrolysis by a factor of ~10. These macrocycles had a much greater effect on the hydrolysis of ATP to ADP and phosphate,

increasing the rate by a factor of ~100.[17] This may be because the macrocycles bind ATP more strongly than they bind pyrophosphate. Binding to the substrate serves to increase the effective concentration of the amine. The same is true for pentaethylenehexamine, which is able to bind ATP and catalyses ATP hydrolysis[18] **(Figure 4.17)**.

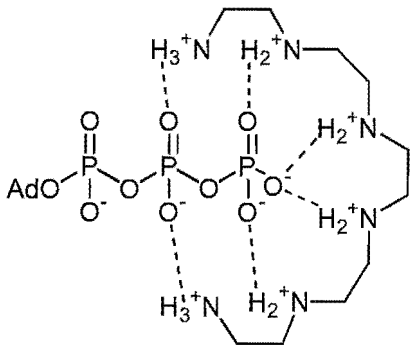


Figure 4.17. Pentaethylenehexamine binding to ATP.

Numerous studies on the hydrolysis of phosphate compounds, such as the hydrolysis of acetyl phosphate and ATP, have shown that most simple amines have no, or only a small effect, on the rate of hydrolysis. Most likely this is partly due to the high concentration of water that amines must compete with as nucleophiles.

The negligible effect of amines on the hydrolysis of polyphosphates is highlighted by the recent findings of Admiraal and Herschlag, who studied the hydrolysis of ATP in the presence of amines.[19] They found that at 95°C amines reacted with ATP ~10-fold faster than water. The authors had to resort to trapping the phosphorylated amine intermediate with fluoride ion to measure the rate constant for the reaction of amines with ATP. This approach was successful since fluoride reacts faster with phosphorylated amines than it does with ATP, and the ratio of fluorophosphate to phosphate was found to increase with increasing amine concentration. The observed increase in the rate of ATP disappearance was very small, since water was present at a concentration of at least 55x that of the amine.

The effect of glycine and imidazole on the hydrolysis of pyrophosphate was first determined in the absence of any added metal ions at pH 6.5 **(Figure 4.18)**. These amines had no significant effect on the rate of pyrophosphate hydrolysis. However, since the concentration of water was ~1000 times greater than the concentration of amine, even if the amines did catalyse hydrolysis, the increase may have been too small to detect.

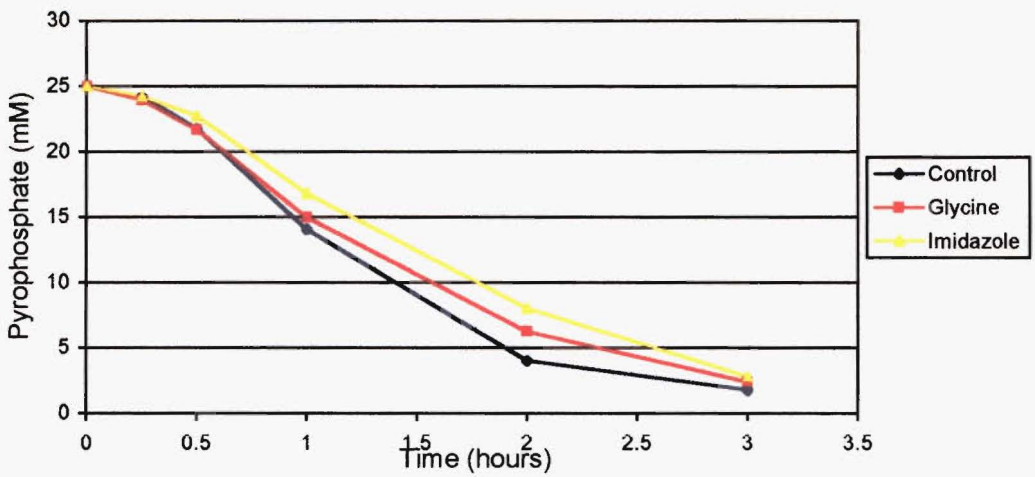


Fig. 4.18. The effect of amines on the hydrolysis of pyrophosphate.

Buffered solutions containing 0.025M pyrophosphate and 0.05M glycine or imidazole were incubated at 95°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with EDTA.

The effect of amines on the hydrolysis of pyrophosphate in the presence of Fe^{2+} was also studied since, in the presence of metal ions, some amines have been shown to affect phosphoryl transfer reactions. The effect of glycine, imidazole and AMP on the hydrolysis of pyrophosphate in the presence of Fe^{2+} is shown below **(Figure 4.19)**.

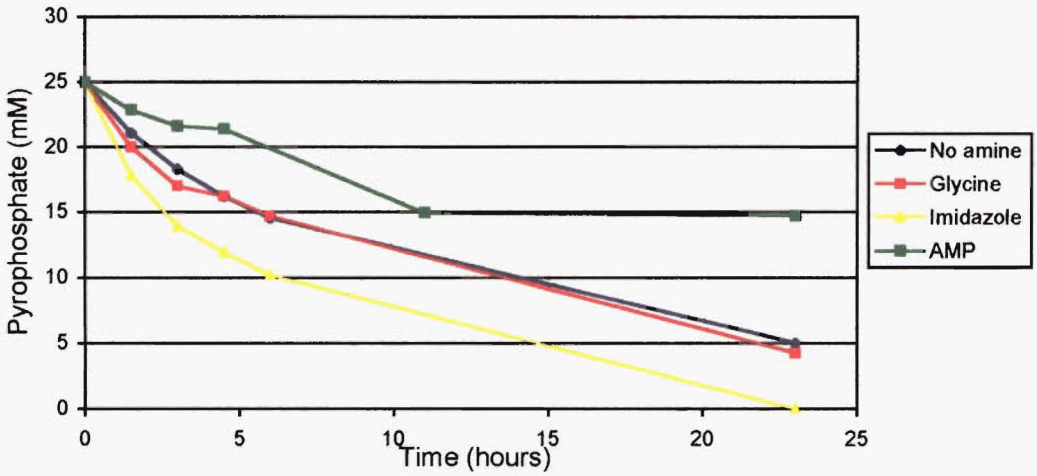


Figure 4.19. Effect of amines on the hydrolysis of pyrophosphate in the presence of Fe(II).

Buffered mixtures containing 0.025M pyrophosphate, 0.05M amine and 0.1M ferrous sulfate were incubated at 95°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with potassium cyanide.

In the presence of Fe^{2+}, imidazole had a significant effect on the rate of pyrophosphate hydrolysis, while glycine had no effect. This is in contrast to the effect of amines in the absence of Fe^{2+}, where both imidazole and glycine had no effect. Glycine may not affect the hydrolysis of pyrophosphate at pH 6.5, since the protonated amine is an ineffective nucleophilic catalyst.

These results suggest that imidazole does catalyse the hydrolysis of pyrophosphate, but under most conditions the rate acceleration due to imidazole is too small to be detected. Imidazole may act in synergy with Fe^{2+} to catalyse pyrophosphate hydrolysis. Alternatively, Fe^{2+} may slow down the hydrolysis of pyrophosphate sufficiently so that any rate acceleration due to imidazole can be detected. The catalysis of pyrophosphate hydrolysis by imidazole is consistent with the finding of Admiraal and Herschlag that imidazole catalyses ATP hydrolysis.

The inhibition of pyrophosphate hydrolysis in the presence of AMP is a little surprising since the purine ring of AMP contains an imidazole-like nitrogen. However AMP coordinates to divalent metal ions through the phosphate oxygens and N(7), which is the imidazole-like nitrogen. This would prevent N(7) from being involved in nucleophilic catalysis. The inhibition of pyrophosphate hydrolysis may be due to the AMP binding to the surface of the Fe-pyrophosphate precipitate. The hydrophobic adenine moiety of AMP could act to further shield the pyrophosphate from the solvent.

4.4 Hydrolysis of adenosine nucleotides in the presence of Fe^{2+}.

Reactions of ADP and ATP in the presence of Fe^{2+} were investigated next. Like pyrophosphate and tripolyphosphate, ADP and ATP exist predominantly as the trianion and the tetraanion, respectively, at neutral pH, as the internal phosphates of both ADP and ATP have pK_a's of about 2, and the terminal phosphates have pK_a's of about 2 and 6.5.[5] A number of studies have previously been conducted on the hydrolysis of these two compounds in solution, because of their biological importance. However, previous studies have focussed on the solution chemistry of ADP and ATP. In the reactions investigated in this study, the concentrations of Fe^{2+} and ADP, or ATP, were quite high, such that precipitates formed. This means that the results obtained here may not be strictly comparable to the results from previous studies.

4.4.1 Hydrolysis of ADP.

Comparing the hydrolysis of ADP in the presence of Fe^{2+} with the hydrolysis of pyrophosphate in the presence of Fe^{2+} may provide an insight into how the presence of the adenosine moiety affects the coordination of Fe^{2+}. This may shed light on how the coordination of Fe^{2+} affects the hydrolysis of these polyphosphates.

Tetas and Lowenstein have investigated the hydrolysis of ADP in the presence of a wide range of divalent metal ions.[20] At pH 9 all the metal ions investigated accelerated ADP hydrolysis, while at pH 5 only Zn^{2+} and Cu^{2+} had a significant effect on the rate of

hydrolysis. However, at pH 5, other divalent metal ions, including Ni^{2+}, Mn^{2+} and Co^{2+} had very little effect on the rate of hydrolysis.

In these studies, the hydrolysis of ADP was carried out at 95°C in reactions buffered at pH 6.5 in the presence, and the absence, of Fe^{2+} **(Figure 4.20)**.

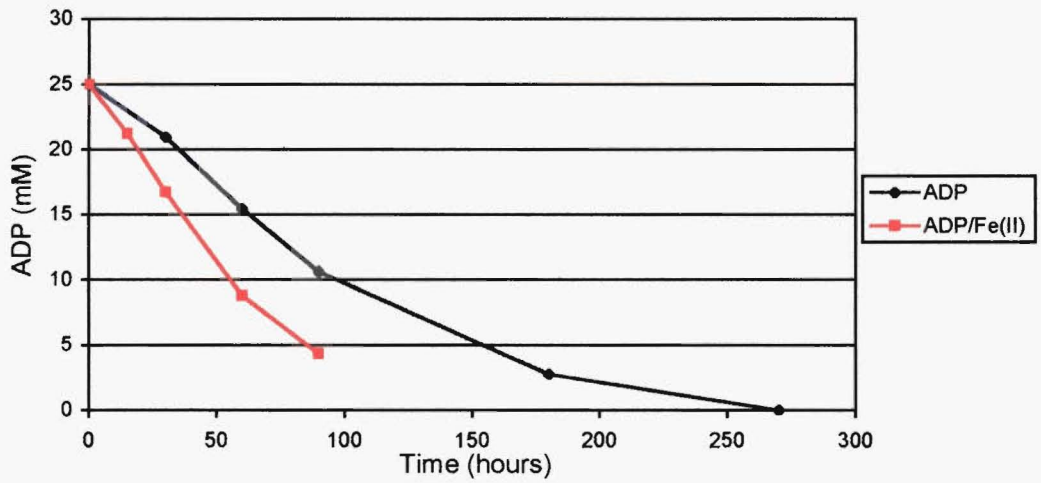


Figure 4.20. The effect of Fe^{2+} on the hydrolysis of ADP.

Buffered reactions containing 0.025M ADP were incubated at 95°C with or without 0.1M ferrous sulfate present. Aliquots were removed and analysed by ^{31}P NMR after treatment with EDTA or potassium cyanide.

The hydrolysis of ADP is accelerated in the presence of Fe^{2+} compared to the control with no Fe^{2+} present. This is in contrast to the situation with inorganic pyrophosphate, where the presence of Fe^{2+} inhibited hydrolysis. This may be due to the different modes of coordination of Fe^{2+} to these two diphosphates. Pyrophosphate predominantly coordinates to divalent metal ions through both phosphates. ADP on the other hand, because of the presence of the adenine ring, exists to a large extent as the macrochelate, with the metal ion bound to the β-phosphate and N(7) **(Figure 4.21)**. The extent of macrochelate formation with Fe^{2+} is not known, however one would expect it to be similar to the extent of macrochelate formation for the Ni^{2+} and Zn^{2+} complexes of ADP, which is about 80% and 67% respectively.[5]

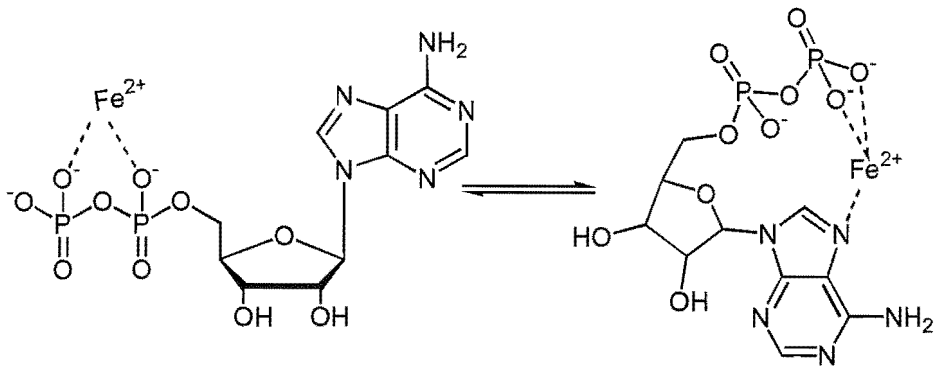


Figure 4.21. Fe^{2+}-ADP complexes.

The macrochelate complex may catalyse ADP hydrolysis since Fe^{2+} binds to the β-phosphate, but not the α-phosphate. The metal ion would reduce the charge density surrounding the phosphates, and may make the β-phosphate more electrophilic. Hydrolysis may also occur intramolecularly, via a Fe^{2+} bound water or hydroxide.

The hydrolysis of ADP could occur through nucleophilic attack at the β-phosphate, with loss of AMP, or via attack at the α-phosphate, with inorganic phosphate as the leaving group. In the case of hydrolysis these two pathways cannot be distinguished since they result in the same products. However, nucleophilic attack would be expected to occur at the β-phosphate. Complexation with the Fe^{2+} would lower the charge density surrounding this phosphate and make it more electrophilic **(Figure 4.22)**.

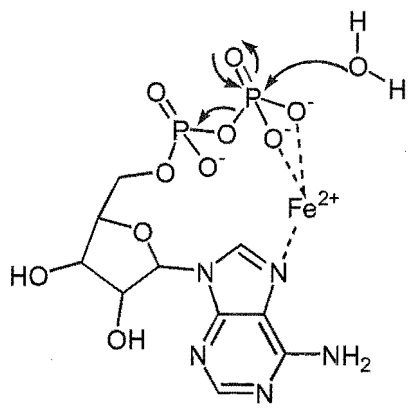


Figure 4.22. Possible mechanism for the hydrolysis of Fe^{2+}-ADP.

Pyrophosphate and ADP would also differ in the type of precipitates they formed. Although both pyrophosphate and ADP formed precipitates in the presence of Fe^{2+}, the Fe^{2+}-ADP complex is expected to be more soluble than the Fe^{2+}-pyrophosphate complex because of the large adenosine group in ADP. The enhanced solubility of the ADP complex might be expected to lead to more hydrolytic chemistry, due to the effective increase in the concentration of water which ADP experiences.

4.4.2 Hydrolysis of ATP

The hydrolysis of ATP is much more complicated than the hydrolysis of any of the other polyphosphates investigated. This is because there are now three different phosphates that could be the subject of nucleophilic attack and two different phosphoanhydride bonds that could be hydrolysed. The reaction profile is also more complex, since the initial products of hydrolysis undergo further hydrolysis.

The hydrolysis of ATP in the presence of metal ions has been extensively investigated as a result of its biological importance. The ability of a metal ion to promote hydrolysis depends in part on the affinity of the metal ion for nitrogen donor ligands. Metal ions that do not bind to N(7), such as Mg^{2+}, have very little effect on the rate of ATP hydrolysis, while metal ions like Cu^{2+} and Zn^{2+} that bind to N(7) promote the hydrolysis of ATP. The promotion of ATP hydrolysis in the presence of metal ions that bind to N(7) has been proposed to be due to the formation of a reactive dimer, involving two ATP molecules and four metal ions.[21] In this case, the metal ion coordinates with the α- and β- phosphates of one ATP and N(7) of the other ATP **(Figure 4.23)**.

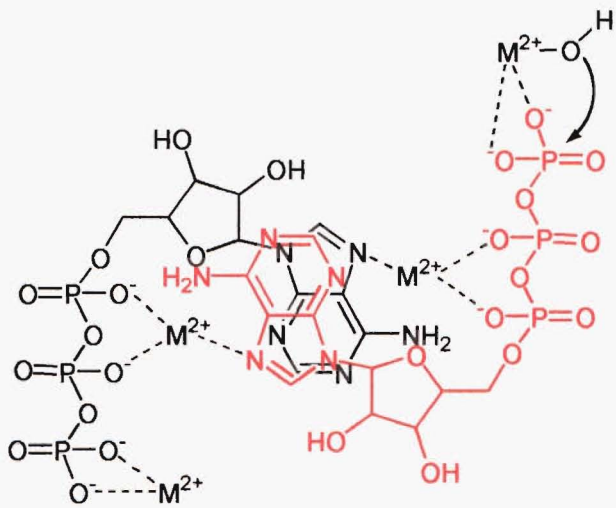


Figure 4.23. Postulated structure for the reactive $[M^{2+}_2(ATP)_2]$ dimer.

The hydrolysis of ATP in aqueous solution at pH 6.5 and at 95°C was monitored first and is shown below **(Figure 4.24)**.

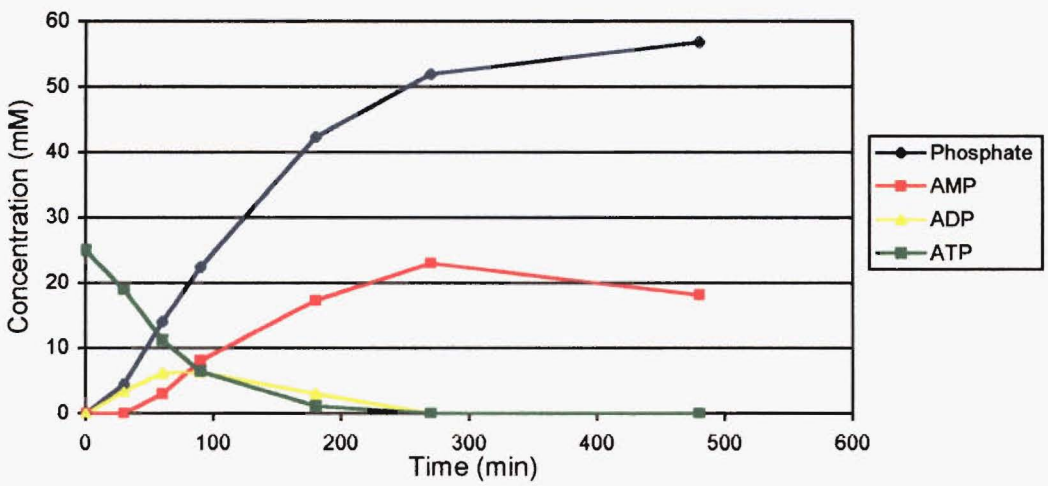


Figure 4.24. Time course for ATP hydrolysis.

A buffered solution containing 0.025M ATP was incubated at 95°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with EDTA.

From the reaction profile, it can be seen that ADP accumulates for a short while and then undergoes further hydrolysis. The formation of AMP also shows a short lag time, which

suggests that it is derived from ADP, and not by direct hydrolysis of ATP. The AMP is also slowly hydrolysed, but at a rate much slower than that of ATP and ADP. This is not surprising since in the hydrolysis of AMP a phosphate ester is hydrolysed, while the hydrolysis of ADP and ATP involve phosphoanhydride hydrolysis. This reaction profile indicates that hydrolysis occurs via the sequential loss of the γ-phosphate, β-phosphate and α-phosphate of ATP **(Figure 4.25)**.

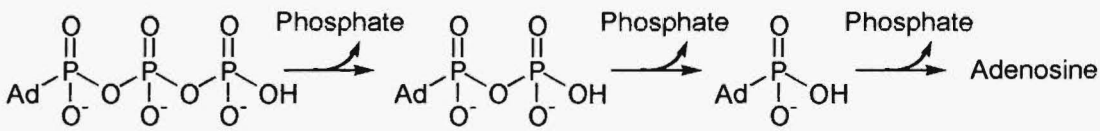


Figure 4.25. Hydrolysis of ATP via the sequential loss of terminal phosphates.

The hydrolysis of ATP was next investigated in the presence of Fe^{2+} and FeS **(Figure 4.26)**.

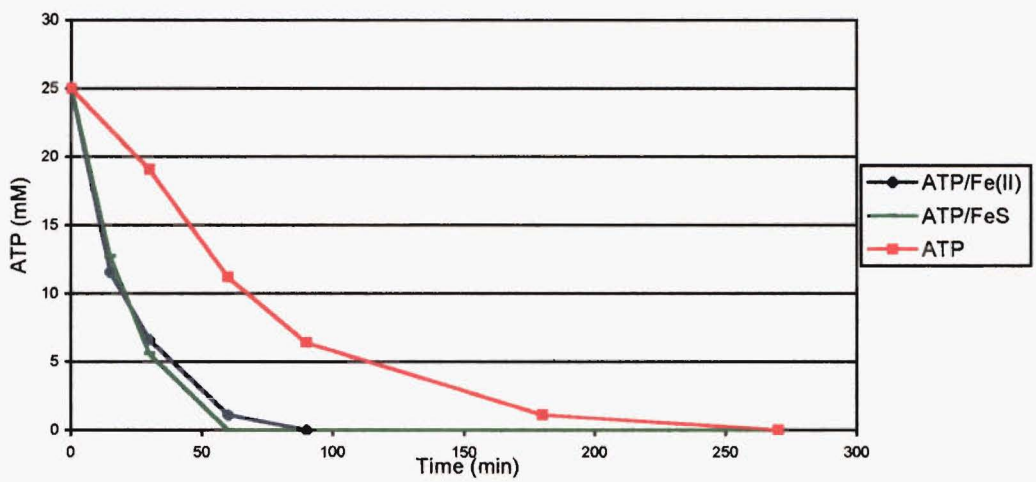


Figure 4.26. The effect of Fe^{2+} and FeS on the hydrolysis of ATP.

Buffered reactions containing 0.025M ATP were incubated at 95°C with or without 0.1M ferrous sulfate or 0.1M ferrous sulfate and 0.1M NaSH present. Aliquots were removed and analysed by ^{31}P NMR after treatment with EDTA or potassium cyanide.

The rate of ATP hydrolysis in the presence of Fe^{2+} or FeS is increased compared to the hydrolysis of ATP in the absence of Fe^{2+} and sulfide. The rate of hydrolysis of ATP is virtually identical in the presence of Fe^{2+} and FeS. When sulfide is present, the ATP presumably binds to Fe^{2+} sites on the surface of FeS or to free Fe^{2+} in solution. The finding that ATP hydrolysis is catalysed by Fe^{2+} is consistent with the results obtained for the hydrolysis of tripolyphosphate, which was also faster in the presence of Fe^{2+}. However the reaction profile for the hydrolysis of ATP in the presence of Fe^{2+} is very different from the reaction profile obtained in the absence of Fe^{2+} **(Figure 4.27)**. The hydrolysis of ATP in the presence of Fe^{2+} produced significant amounts of pyrophosphate, which was not seen with ATP hydrolysis in the absence of Fe^{2+}. ATP hydrolysis to produce pyrophosphate was also observed when FeS was present **(Figure 4.28)**.

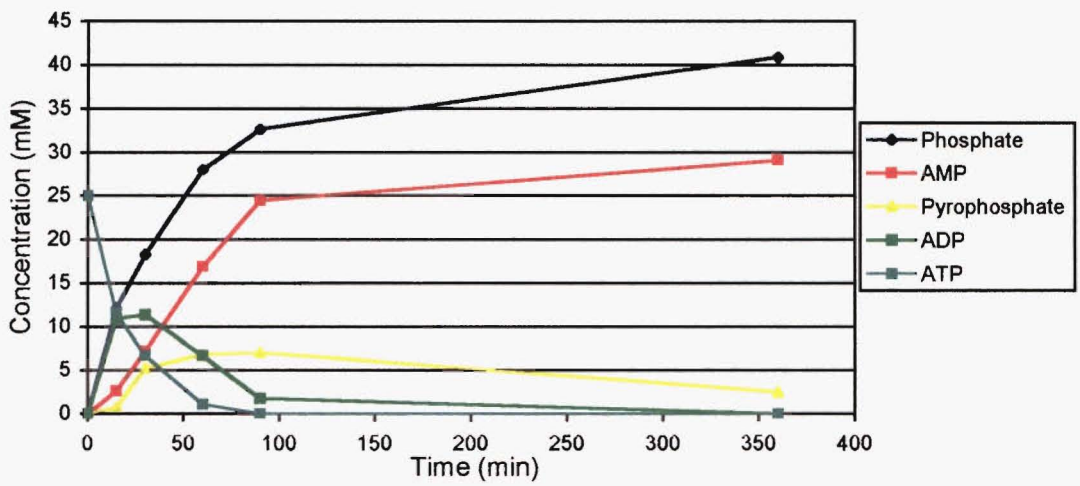


Figure 4.27. Time course for ATP hydrolysis in the presence of Fe^{2+}.

A buffered mixture containing 0.025M ATP and 0.1M ferrous sulfate was incubated at 95°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with potassium cyanide.

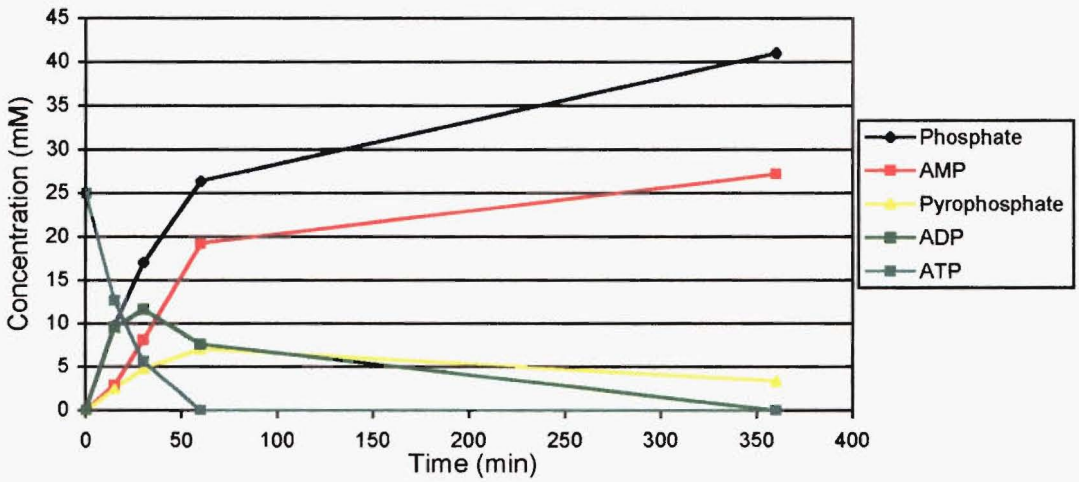


Figure 4.28. Time course for ATP hydrolysis in the presence of FeS.

A buffered mixture containing 0.025M ATP, 0.1M ferrous sulfate and 0.1M NaSH was incubated at 95°C. Aliquots were removed and analysed by ^{31}P NMR after treatment with potassium cyanide.

There are two possible mechanisms that could lead to the formation of pyrophosphate: hydrolysis to produce AMP and pyrophosphate; or phosphorolysis with inorganic phosphate that is produced during the hydrolysis of ATP **(Figure 4.29)**.

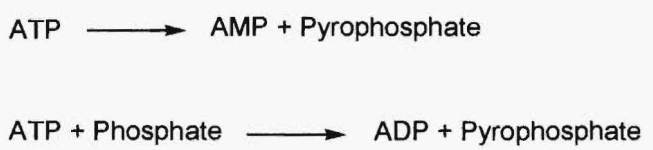


Figure 4.29. Two pathways leading to pyrophosphate formation.

Support for both pathways can be found in the literature.[20,22] However, since the initial formation of AMP is observed to be about the same rate as production of pyrophosphate, it is likely that pyrophosphate arises through the hydrolysis of ATP, and not through phosphorolysis.

The types of complexes that Fe^{2+} forms with ATP are not known, but the most likely complexes can be inferred from other M^{2+}-ATP complexes. Divalent metal ions coordinate predominantly to the triphosphate chain of ATP, although coordination to N(7) of adenine also occurs. When the metal ion coordinates to N(7), the α-phosphate no longer coordinates to the metal ion.[6] The extent of macrochelate formation in M^{2+}-ATP complexes is less than that in M^{2+}-ADP complexes, but is still significant. For instance, 56% of the Ni^{2+}-ATP complex is the macrochelate, while 28% of the Zn^{2+}-ATP complex is the macrochelate.[5] One would expect that Fe^{2+} would also form a macrochelate to a significant extent **(Figure 4.30)**.

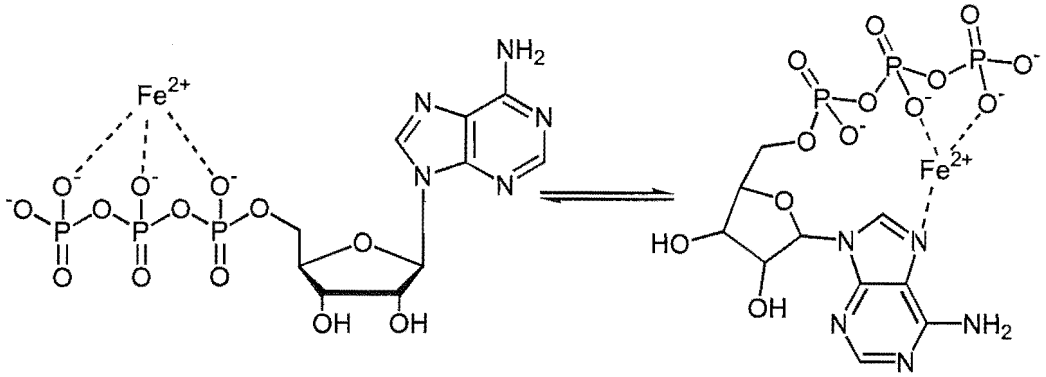


Figure 4.30. Possible Fe^{2+}-ATP complexes.

These two complexes may explain the reactivity of ATP in the presence of Fe^{2+}. Coordination of Fe^{2+} to the phosphates of ATP would be similar to the complex formed between tripolyphosphate and Fe^{2+}. In the presence of an excess of Fe^{2+}, a second Fe^{2+} ion may also be bound to the terminal phosphate, with the first Fe^{2+} being bound to the α- and β- phosphates. This could make the phosphoanhydride bond between the β- and γ- phosphates more susceptible to hydrolysis. This has been postulated to be the case for the hydrolysis of ATP in the presence of other divalent metal ions[23]. However, the active species is believed to be a dimer $[M^{2+}{}_2(ATP)_2]$,[21] with a metal ion co-ordinating the α- and β- phosphates of one ATP and N(7) of the second ATP (see Figure 4.23).

The production of pyrophosphate could be due to the formation of the Fe^{2+}-ATP macrochelate complex. In the macrochelate complex the Fe^{2+} would be bound to the β- and γ- phosphates of ATP, in addition to N(7) of adenine. This may make the α,β-phosphoanhydride bond more susceptible to hydrolysis, and would lead to the formation of pyrophosphate and AMP **(Figure 4.31)**.

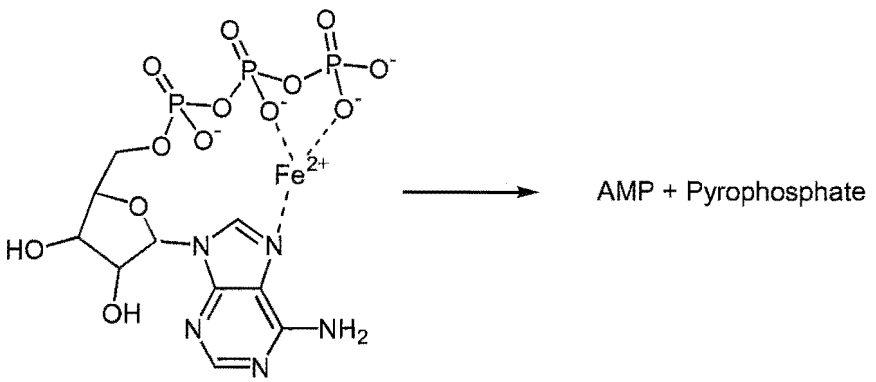


Figure 4.31. Possible Fe^{2+}-ATP complex leading to the formation of pyrophosphate.

The formation of pyrophosphate is interesting because it highlights the versatility of ATP, as opposed to ADP. Pyrophosphate formation could occur by nucleophilic attack at the β-phosphate, with expulsion of AMP, or attack at the α-phosphate with pyrophosphate as the leaving group **(Figure 4.32)**.

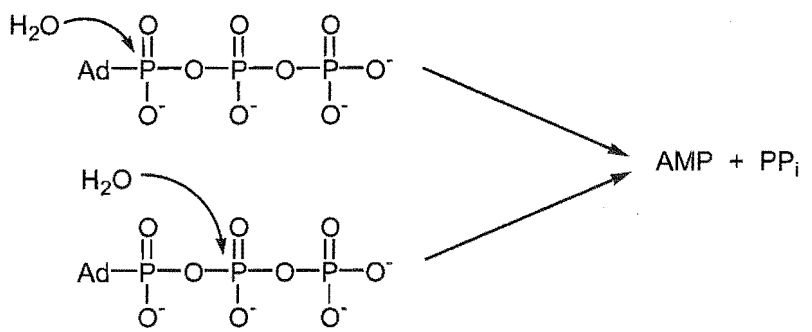


Figure 4.32. Mechanisms of ATP hydrolysis to form pyrophosphate and AMP.

The two pathways cannot be distinguished from the products of hydrolysis since the products are identical. Complexes involving the α- and β- phosphates of ATP, or the β- and γ- phosphate could be involved in pyrophosphate formation. A third possibility is one where the 3′-OH of ATP attacks the α-phosphorus, leading to the formation of 3′,5′-cyclic AMP. This would then be rapidly hydrolysed to AMP **(Figure 4.33)**. This mechanism may occur when Fe^{2+} coordinates to the phosphates only, but is unlikely to be operating in the macrochelate due to steric constraints since coordination of Fe^{2+} to N(7) will prevent the 3′-OH from approaching the α-phosphate (see Fig 4.31).

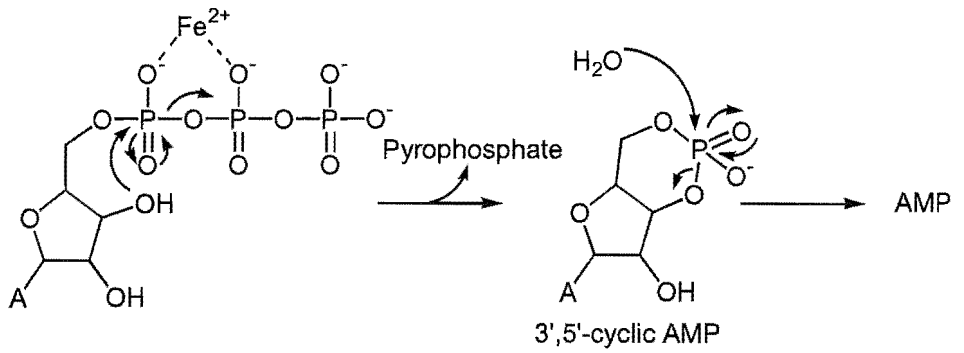


Figure 4.33. Hydrolysis of ATP via intramolecular attack of the 3′-OH.

As stated above, the hydrolysis of ATP to generate pyrophosphate and AMP could occur via two distinct pathways, involving attack at the β-phosphorus or the α-phosphorus. Nucleophilic attack at the β-phosphorus results in pyrophosphoryl transfer, while attack at the α-phosphorus leads to adenylyl transfer **(Figure 4.34)**.

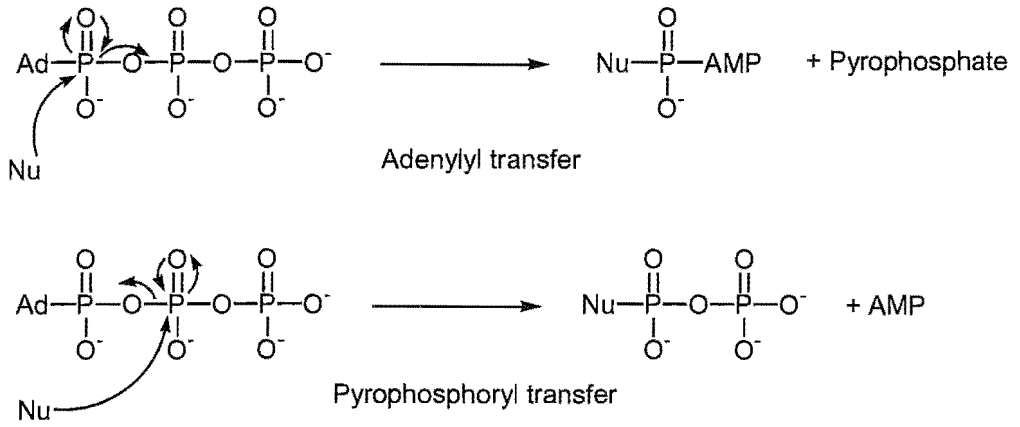


Figure 4.34. Adenylyl and pyrophosphoryl transfer.

When water is the nucleophile these two pathways can not be distinguished, as the products are identical. If another nucleophile were used, the two pathways would be able to be distinguished since the products would be different. Unfortunately, attack by nucleophiles other than water has so far not been observed, although others have reported phosphoryl transfer to various nucleophiles, including acetate and glycine,[24] in the presence of divalent metal ions (see section 4.4).

The versatility of ATP is exploited by nature. Enzyme catalysed reactions of ATP, and other nucleotide triphosphates, can occur via a number of different mechanisms, based on the phosphorus centre that is attacked by the nucleophile. The widespread use of adenylyl transfer in biology, compared with the rarity of pyrophosphoryl transfer, suggests that the formation of pyrophosphate in the presence of Fe^{2+} may be due to nucleophilic attack at the α-phosphate.

4.5 Attempts to Catalyse Phosphoryl Transfer to Nucleophiles other than Water.

The hydrolysis of polyphosphates results in a loss of free energy, since the energy released through hydrolysis is lost as heat. To utilise the free energy of hydrolysis of

polyphosphates requires a nucleophile other than water, so that some of the energy contained in the phosphoanhydride bond is retained in the new bond formed.

The non-enzymatic phosphorylation of various nucleophiles using ATP has been demonstrated previously. In the presence of divalent metal ions at pH 9, ATP was shown to phosphorylate inorganic phosphate.[22] The most effective catalysts for this reaction were Mn^{2+}, Cd^{2+} and Ca^{2+}. Interestingly Fe^{2+} was also found to catalyse the reaction, although at about $1/10^{th}$ of the rate of the other metal ions. The pH optimum of this reaction was at a pH of 9 and was quite sharp, with very little pyrophosphate being formed at pH 7.

Phosphoryl transfer from ATP to acetate, glycine and β-alanine has also been reported.[24] The acyl phosphates that form are not stable, so they were trapped with hydroxylamine to form hydroxamates **(Figure 4.35)**.

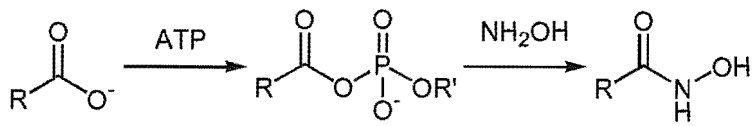


Figure 4.35. Hydroxylamine trapping of an acyl phosphate.

In this case, Be^{2+} was the most effective catalyst found, with Ni^{2+} and Zn^{2+} also catalysing the reaction. The optimum pH was 5.2, indicating the carboxylic acid was the nucleophile.

Glucose and adenosine were chosen as potential nucleophiles for phosphoryl transfer from pyrophosphate. The expected products of phosphorylation, glucose-6-phosphate and AMP **(Figure 4.36)** are stable under the conditions used, so their presence would be observable using ^{31}P NMR.

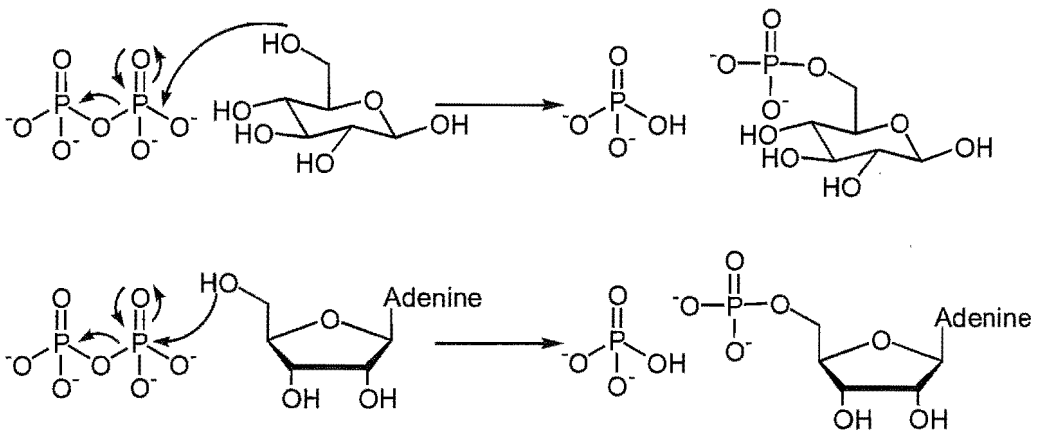


Figure 4.36. Phosphoryl transfer to glucose and adenosine.

However, phosphoryl transfer to glucose or to AMP was not observed under the conditions used. A possible reason for this may be that the nucleophiles (glucose and adenosine) do not interact with the precipitate very well. If this were the case, then phosphoryl transfer to glucose or adenosine would be unlikely, as the pyrophosphate is sequestered within the precipitate while the nucleophiles remain in solution. An important future goal for this research is to show that phosphoryl transfer from pyrophosphate to nucleophiles other than water can take place. The chemistry of ATP also demands further attention, particularly with respect to the ability of ATP to undergo phosphoryl, pyrophosphoryl and adenylyl transfer. Under what conditions do these reactions take place?

4.6 Chapter Summary.

Pyrophosphate was found to be more stable towards hydrolysis in the presence of Fe^{2+} and Ni^{2+} than in the absence of either metal ion. The protecting influence of these metal ions may be due to the formation of a chelate, or to the formation of a metal ion-pyrophosphate precipitate. The ability of Fe^{2+} and Ni^{2+} to stabilise pyrophosphate towards hydrolysis may have important implications for the origins of

life, suggesting that significant amounts of pyrophosphate may have accumulated in the presence of these metals, for example near hydrothermal vents.

Amines had no affect on the hydrolysis of pyrophosphate in the absence of metal ions. However, in the presence of Fe^{2+}, imidazole was shown to catalyse pyrophosphate hydrolysis, while AMP inhibited pyrophosphate hydrolysis.

In contrast to the hydrolysis of pyrophosphate, Fe^{2+} catalysed the hydrolysis of tripolyphosphate, ATP and ADP. Catalysis of tripolyphosphate and ATP hydrolysis may have been due to two metal ions binding to the substrate. A possible reason why the hydrolysis of ADP was catalysed by Fe^{2+}, but pyrophosphate hydrolysis was inhibited by Fe^{2+}, is the different complexes formed with ADP because of the presence of the adenine ring.

In the presence of Fe^{2+}, ATP was found to hydrolyse by two different pathways. The major pathway was hydrolysis of the terminal phosphate, to form ADP and inorganic phosphate. The second pathway resulted in the formation of AMP and pyrophosphate. The versatility of ATP with respect to phosphoryl transfer may have been a reason for its importance in biological systems.

Attempts to catalyse phosphoryl transfer from pyrophosphate to glucose or AMP proved unsuccessful under the conditions used. The investigation of phosphoryl transfer to nucleophiles other than water is an important goal for future research.

4.7 References for Chapter Four.

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5 Experimental.

5.1 General Methods.

Unless otherwise stated, all chemicals were obtained from commercially available sources and were generally of analytical grade.

Nuclear magnetic resonance (NMR) spectroscopy was performed on either a Varian Unity 300 instrument, or on a Varian XL 300 instrument. These instruments operate at 300MHz for ^{1}H nuclei and at 120MHz for ^{31}P nuclei. The probe temperature for all the experiments was 23°C. Peak positions are quoted relative to a reference peak. This was the methyl protons of methanol for ^{1}H spectra, or a sample of 85% phosphoric acid in D_2O for ^{31}P spectra. ^{31}P NMR spectroscopy was obtained used a 5 second delay time between acquisitions, and data was collected for 40 transients.

Electron ionisation (EI) and fast atom bombardment (FAB) mass spectroscopy was performed using a Kratos MS80 mass spectrometer operating at 4kV.

High-speed centrifugation was performed using an Eppendorf Centrifuge 5403. Samples of less than 1.5mL volume were centrifuged at up to 15,000 r.p.m., and on a larger scale at up to 5,000 r.p.m.

Absorbance measurements were performed using a Pharmacia spectrophotometer operating at a fixed wavelength.

Thin-layer chromatography (TLC) was performed using either Merck DC-Plastikfolien Kieselgel 60 F_{254} silica plates or Merck DC-Plastikfolien cellulose plates. Iodine, 2,4-DNP spray or UV illumination was used to visualise organic compounds.

5.1.1 General method for the reactions involving phosphate compounds and ferrous ions.

All experiments requiring Fe^{2+} were performed under an atmosphere of argon using syringe techniques. Solvents were degassed by bubbling argon through them for 1 hour and were stored under argon. All reagents were typically AR grade and used without further purification. Reactions were initiated by adding a solution of $FeSO_4$ to a buffered solution containing all other reagents, followed by heating. The amounts of each reagent used in the reactions are expressed as the concentrations that would be present if no precipitation occurred. Aliquots (typically 400μL) were withdrawn via syringe at various time intervals and added to KCN (~12eq CN^-/Fe^{2+}) in 100μL D_2O. Samples were centrifuged to remove solid material before being analysed by ^{31}P NMR. Experiments were performed at least twice, and the results were averaged.

5.2 Experimental for Chapter 2: Acetyl Phosphate Chemistry.

5.2.1 Synthesis of dilithium acetyl phosphate.[1]

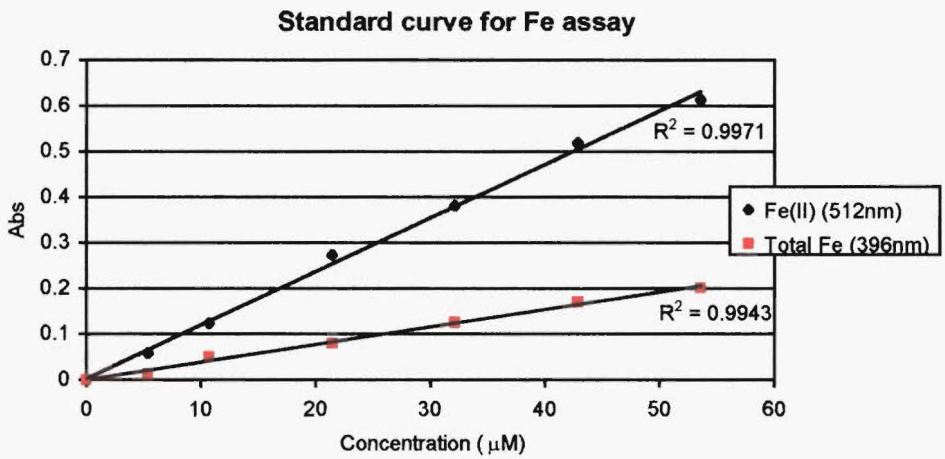
Pyridine (95g, 1.2mol) and a solution of dipotassium hydrogen phosphate (0.25M, 200mL) were stirred at 0°C in a 500mL flask. Acetic anhydride (9.6mL, 0.1mol) was added dropwise over 5 minutes. After 30 minutes, lithium hydroxide (4M, 38mL) was added. Cold ethanol (2300mL) was added slowly and after standing for 2 hours at 0°C the white precipitate was filtered, washed with ethanol and ether, and air-dried. The white solid was dissolved in water (80mL), and then cold ethanol (40mL) was added. This was filtered, and cold ethanol (400mL) was added slowly to the filtrate. After 2 hours at 0°C, the white precipitate was collected, washed with ethanol and ether, and dried *in vacuo* to give 5.01g (66%) of dilithium acetyl phosphate.

δ ^{1}H NMR (D_2O): 2.06 (s, $C\underline{H}_3$)

δ ^{31}P NMR(D_2O): -0.95.

5.2.2 Assay for [Fe^{2+}] and total [Fe].[2]

A solution of $FeNH_4(SO_4)_2.7H_2O$ (0.5mg/ml) was used to construct a standard curve. An aliquot of the Fe standard was diluted to 1mL using ~5mM H_2SO_4, and 2mL of a fresh 10% solution of hydroxylamine hydrochloride was added. The solution was adjusted to pH 3-4 with a 10% sodium citrate solution, and then 5mL of 0.25% phenanthroline solution was added. The solution was made up to a final volume of 50mL and after 5 minutes the absorbance at 512nm and at 396nm was measured.



Least squares regression of the standard curves gave the following equations to determine the concentration of Fe^{2+} and total Fe.

$$[Fe^{2+}] = \frac{(Abs^{512} - 0.00475) \times 10^{-6}}{0.0117}$$

$$[Fe]_{total} = \frac{(Abs^{396} + 0.000388) \times 10^{-6}}{0.00386}$$

5.2.3 Determination of the level of oxidation in a typical experimental.

A mixture containing MES buffer (0.5M, pH 6.5), acetyl phosphate (0.05M), phosphate (0.05M) and ferrous sulphate (0.075M) was incubated at 50°C. Aliquots were removed after 5, 10, 20, 30 and 60 minutes. 15μL aliquots of the remaining mixture were assayed for Fe^{2+} and total Fe using the above method, except that the final volume was 25mL.

	Abs	[ ] of 25mL	[ ] of 15μL aliquot
Fe^{2+} (λ_{512})	0.538	45.58μM	75.96mM
Total Fe (λ_{396})	0.181	49.99μM	78.32mM

5.2.4 Effect of pH on the Fe(II) catalysed formation of pyrophosphate from acetyl phosphate and phosphate.

Mixtures containing buffer (0.5M), phosphate (0.05M), acetyl phosphate (0.05M) and ferrous sulfate (0.05M) were prepared at 0°C and incubated at 38°C. Aliquots were withdrawn at various times, treated with potassium cyanide and analysed by ^{31}P NMR. MES was used to buffer at pH 5.5, 6.0 and 6.5, PIPES at pH 7.0, and HEPES at pH 7.5.

pH 5.5

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
10	49.61	46.85	1.78
20	58.57	37.55	1.95
30	68.03	28.61	1.69
60	84.06	11.74	2.10
90	90.65	6.55	1.45

pH 6.0

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
10	57.11	35.60	3.65
20	61.46	29.94	4.30
30	69.73	22.01	4.14
60	77.75	12.85	4.70
90	85.66	6.630	3.86

pH 6.5

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
10	58.49	31.33	5.10
20	62.29	24.53	6.59
30	66.48	18.44	7.55
60	75.14	9.165	7.86
90	81.11	4.865	7.02

pH 7.0

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
10	60.69	29.18	5.07
20	67.93	19.92	6.08
30	73.30	15.34	5.68
60	80.10	7.75	6.08
90	83.01	4.25	6.37

pH 7.5

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
10	58.16	36.18	2.83
20	63.17	29.37	3.73
30	65.86	25.34	4.40
60	75.55	12.61	5.92
90	84.26	6.57	4.58

5.2.5 Effect of Fe(II) concentration on the formation of pyrophosphate from acetyl phosphate and phosphate.

Mixtures containing MES buffer (0.5M, pH 6.5), phosphate (0.05M), acetyl phosphate (0.05M) and ferrous sulfate (0.025M, 0.05M, 0.0625M, 0.075M or 0.1M) were prepared at 0°C and incubated at 38°C. Aliquots were withdrawn at various times, treated with potassium cyanide and analysed by ^{31}P NMR.

0.025M $FeSO_4$

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
60	53.74	46.25	0.00
150	69.17	30.83	0.00
310	81.36	18.64	0.00

0.05M $FeSO_4$

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
15	49.90	45.15	2.47
30	56.92	35.02	4.03
60	62.87	28.13	4.50
120	70.52	19.56	4.97
180	72.80	18.58	4.34
240	80.38	8.87	5.38

0.0625M $FeSO_4$

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
5	53.55	43.34	1.56
10	51.97	41.42	3.31
20	54.60	35.15	5.13
30	57.38	30.20	6.21
60	62.44	22.93	7.31
130	71.58	13.21	7.61
310	78.70	0.88	10.23

0.075M $FeSO_4$

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
5	57.82	39.35	1.42
10	55.36	34.22	5.22
20	60.00	23.76	8.12
30	63.83	16.59	9.79
60	65.65	10.02	12.17
120	71.35	2.34	13.16
210	74.90	0.00	12.55

0.1M $FeSO_4$

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
5	53.97	43.04	1.49
10	62.28	27.38	5.17
20	67.59	14.53	8.94
30	68.79	9.14	11.04
60	72.66	3.20	12.07
120	75.44	0.00	12.28

5.2.6 The effect of temperature on the Fe(II) catalysed formation of pyrophosphate from acetyl phosphate and phosphate.

Mixtures containing MES buffer (0.5M, pH 6.5), phosphate (0.05M), acetyl phosphate (0.05M) and ferrous sulfate (0.075M) were prepared at 0°C and incubated at either 22°C, 38°C, 51°C or 67°C. Aliquots were withdrawn at various times, treated with potassium cyanide and analysed by ^{31}P NMR.

22°C

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
60	67.47	25.25	3.65
120	72.30	18.90	4.41
180	73.73	15.25	5.52
300	76.95	9.29	6.88
420	79.63	6.10	7.14

38°C

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
5	57.82	39.35	1.42
10	55.36	34.22	5.22
20	60.00	23.76	8.12
30	63.83	16.59	9.79
60	65.65	10.02	12.17
120	71.35	2.34	13.16
210	74.90	0.00	12.55

51°C

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
5	52.27	44.16	1.79
10	63.56	23.76	6.34
20	69.71	10.81	9.74
30	72.99	6.59	10.21
90	79.56	0.00	10.22

67°C

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
2.5	63.15	33.17	1.84
5	76.76	18.68	2.29
10	86.44	7.89	2.84
20	93.79	0.65	2.78
90	97.16	0.00	1.42

5.2.7 Effect of amines on the Fe(II) catalysed formation of pyrophosphate from acetyl phosphate and phosphate at 38°C.

Mixtures containing MES buffer (0.5M, pH 6.5), phosphate (0.05M), acetyl phosphate (0.05M), amine (0.05M) and ferrous sulfate (0.075M) were prepared at

0°C and incubated at 38°C. Aliquots were withdrawn at various times, treated with potassium cyanide and analysed by ^{31}P NMR. The amines studied were glycine, imidazole, pyridine and adenosine.

Glycine

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
15	67.37	25.00	3.82
30	73.65	16.01	5.18
60	74.18	9.88	7.98
120	79.08	4.18	8.37
180	83.57	0.95	7.74

Imidazole

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
15	70.83	22.90	3.89
30	76.73	12.02	5.63
60	78.19	6.90	7.46
120	86.24	0.00	6.89
180	85.09	0.00	7.46

Pyridine

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
15	71.35	20.88	3.89
30	76.81	13.34	4.94
60	78.37	7.23	7.22
120	81.08	1.73	8.60
180	81.23	1.44	8.67

Adenosine

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
15	74.32	17.02	4.34
30	77.15	10.82	6.02
60	81.51	5.11	6.70
120	83.36	0.90	7.89
180	85.99	0.00	7.01

5.2.8 Effect of amines on the Fe(II) catalysed formation of pyrophosphate from acetyl phosphate and phosphate at 51°C.

Mixtures containing MES buffer (0.5M, pH 6.5), phosphate (0.05M), acetyl phosphate (0.05M), amine (0.05M) and ferrous sulfate (0.075M) were prepared at 0°C and incubated at 51°C. Aliquots were withdrawn at various times, treated with potassium cyanide and analysed by ^{31}P NMR. The amines studied were glycine, imidazole, pyridine and adenosine.

Glycine

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
5	55.12	34.67	5.11
10	63.74	19.34	8.41
20	67.38	10.60	11.01
30	75.17	4.23	10.30
90	79.88	0.00	10.06

Imidazole

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
5	43.55	52.94	1.75
10	56.88	38.76	2.18
20	72.02	22.88	2.55
30	78.84	15.54	2.87
90	95.56	0.79	1.83

Pyridine

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
10	61.04	35.73	1.62
20	68.77	27.65	1.80
30	77.25	16.59	3.08
60	86.62	7.83	2.78
120	91.32	1.11	3.09

Adenosine

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
5	54.88	43.38	0.82
10	61.96	33.18	2.44
20	75.86	19.32	2.42
30	83.64	13.94	1.21
90	90.80	4.58	2.17

5.2.9 Pyrophosphate formation from acetyl phosphate and phosphate in the presence of potassium ferrocyanide.

A solution containing MES buffer (0.5M, pH 6.5), acetyl phosphate (0.05M), phosphate (0.05M) and potassium ferrocyanide (0.075M) was prepared and incubated at 38°C under an atmosphere of argon. After 3 and 6 hours, 400μL aliquots were withdrawn and analysed by ^{31}P NMR.

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
180	84.56	15.44	0.00
360	95.54	4.46	0.00

5.2.10 Pyrophosphate formation from acetyl phosphate and phosphate in the presence of Fe(II) and EDTA.

A mixture containing MES buffer (0.5M, pH 6.5), acetyl phosphate (0.05M), phosphate (0.05M), EDTA (0.075M) and ferrous sulfate (0.075M) was prepared and incubated at 38°C under an atmosphere of argon. 400μL aliquots were withdrawn after 3 hours and 6 hours, treated with potassium cyanide, and analysed by ^{31}P NMR.

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
180	86.41	13.59	0.00
360	97.02	2.98	0.00

5.2.11 Pyrophosphate formation from acetyl phosphate and phosphate catalysed by ferrous sulfide.

A mixture containing MES buffer (0.5M, pH 6.5), acetyl phosphate (0.05M), phosphate (0.05M), sodium hydrogen sulfide (0.1M) and ferrous sulfate (0.1M) was prepared and incubated at 38°C under an atmosphere of argon. Aliquots were withdrawn at various intervals, treated with potassium cyanide, and analysed by ^{31}P NMR.

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
30	63.74	31.31	2.48
60	82.21	9.52	4.14
120	85.88	2.86	5.64
180	87.55	0.00	6.23

5.2.12 Catalysis of pyrophosphate formation by Ni(II) and Zn(II).

Mixtures containing MES buffer (0.5M, pH 6.5), phosphate (0.05M), acetyl phosphate (0.05M) and nickel or zinc sulfate (0.1M) were prepared and incubated at

38°C. 400μL aliquots were removed at various times, treated with potassium cyanide (12eq CN^-/M^{2+}) in 100μL D_2O, and analysed by ^{31}P NMR.

Ni^{2+}

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
10	64.75	35.25	0.00
20	71.52	28.48	0.00
30	74.77	22.77	0.99
60	83.16	13.95	1.45
120	89.10	6.36	2.28

Zn^{2+}

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM
10	79.73	19.46	0.41
20	85.28	12.49	1.12
30	88.04	9.96	1.01
60	92.14	4.38	1.74
120	94.85	1.82	1.68

5.2.13 Reaction of acetyl phosphate with phosphate acceptors other than inorganic phosphate in the presence of Fe(II).

Mixtures containing MES buffer (0.5M, pH 6.5), phosphate acceptor (0.05M), acetyl phosphate (0.05M) and ferrous sulfate (0.075M) were prepared and incubated at 38°C. Aliquots were removed at various times, treated with potassium cyanide, and analysed by ^{31}P NMR. The phosphate acceptors studied were pyrophosphate, adenosine monophosphate (AMP) and adenosine diphosphate (ADP).

Pyrophosphate

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM	[PPPi]/mM
30	5.09	20.89	36.96	0.00
100	9.97	15.3	37.37	0.00
255	20.87	9.19	34.98	0.00
450	22.87	2.69	37.22	0.00

Adenosine monophosphate.

Time (min)	[AMP]/mM	[Pi]/mM	[AcPi]/mM	[PPi]/mM	[ADP]/mM
10	59.60	6.20	34.10	0.00	0.00
20	60.10	10.70	29.20	0.00	0.00
30	57.00	13.40	26.60	1.50	0.00
60	57.80	17.50	21.40	1.65	0.00
120	57.50	26.90	11.20	2.25	0.00
240	60.50	30.70	3.70	2.60	0.00
300	57.80	33.30	1.80	2.50	0.00

Adenosine diphosphate.

Time (min)	[Pi]/mM	[AcPi]/mM	[PPi]/mM	[ADP]/mM	[ATP]/mM
0	2.96	43.02	0.00	54.02	0.00
120	25.93	18.03	0.00	56.03	0.00
240	32.26	5.39	0.00	62.35	0.00

5.2.14 The reaction of acetyl phosphate in the presence of Fe(II), phosphate and AMP

A mixture containing MES buffer (0.5M, pH 6.5), containing phosphate (0.05M), acetyl phosphate (0.05M), AMP (0.05M) and ferrous sulfate (0.075M) was incubated at 38°C under an argon atmosphere. Aliquots were removed at various times, treated with potassium cyanide, and analysed by ^{31}P NMR.

Time (min)	[AMP]/mM	[Pi]/mM	[AcPi]/mM	[PPi]/mM
10	59.40	48.75	39.60	1.05
20	55.20	54.15	35.25	2.70
30	64.80	50.70	28.35	3.00
60	63.00	58.95	20.25	3.90
90	62.25	60.60	20.25	3.45
220	66.90	68.40	7.05	3.83

5.3 Experimental for Chapter 3: Phosphoenolpyruvate chemistry.

5.3.1 Effect of pH on the Fe(II)-catalysed formation of pyrophosphate from phosphoenolpyruvate and phosphate.

Mixtures containing buffer (0.5M), phosphoenolpyruvate (0.05M), phosphate (0.05M) and ferrous sulfate (0.1M) were incubated at 45°C. Aliquots were withdrawn at various intervals, treated with potassium cyanide and analysed by ^{31}P NMR. Reactions were carried out at pH 6.0 and 6.5 using MES buffer and at pH 7.0 and 7.5 using MOPS buffer.

pH 6.0

Time (day)	[Pi]/mM	[PEP]/mM	[PPi]/mM
1	64.66	30.64	2.35
2	73.31	18.00	4.35
3	76.94	13.39	4.84
4	81.18	9.32	4.75
7	87.79	2.78	4.71
10	94.17	0.00	2.92

pH 6.5

Time (day)	[Pi]/mM	[PEP]/mM	[PPi]/mM
1	62.38	26.63	5.50
2	73.37	13.40	6.61
3	75.84	9.59	7.29
4	78.20	7.30	7.25
7	81.94	4.61	6.73
10	83.08	3.70	6.61

pH 7.0

Time (day)	[Pi]/mM	[PEP]/mM	[PPi]/mM
1	50.27	37.59	6.07
2	65.72	23.18	5.55
3	77.59	11.41	5.50
4	83.23	7.75	4.51
7	85.23	5.70	4.54

pH 7.5

Time (day)	[Pi]/mM	[PEP]/mM	[PPi]/mM
1	53.83	36.52	4.83
2	65.20	22.55	6.13
3	71.44	16.22	6.17
4	74.98	12.87	6.07
7	84.41	5.97	4.82
10	88.50	2.13	4.69

5.3.2 Effect of the concentration of Fe(II) on the formation of pyrophosphate from phosphoenolpyruvate and phosphate.

Mixtures containing MES buffer (0.05M, pH 6.5), phosphate (0.05M), phosphoenolpyruvate (0.05M) and ferrous sulfate (0.025M, 0.05M, 0.075M or 0.1M) were incubated at 45°C. Aliquots were withdrawn at various times, treated with potassium cyanide and analysed by ^{31}P NMR.

0.025M $FeSO_4$

Time (day)	[Pi]/mM	[PEP]/mM	[PPi]/mM
1	63.00	35.81	0.00
2	75.87	24.13	0.00
3	84.26	15.74	0.00
4	92.38	7.62	0.00
6	98.33	1.67	0.00

0.05M $FeSO_4$

Time (day)	[Pi]/mM	[PEP]/mM	[PPi]/mM
1	65.12	33.66	0.00
2	78.74	20.10	0.00
3	87.56	12.44	0.00
4	93.40	6.60	0.00
6	96.58	3.42	0.00

0.075M $FeSO_4$

Time (day)	[Pi]/mM	[PEP]/mM	[PPi]/mM
1	66.47	30.54	1.44
2	79.77	16.57	1.83
3	84.14	11.38	2.24
4	86.65	8.72	2.32
7	89.48	6.20	2.16
10	97.94	0.00	1.05

0.1M $FeSO_4$

Time (day)	[Pi]/mM	[PEP]/mM	[PPi]/mM
1	62.38	26.63	5.50
2	73.37	13.40	6.61
3	75.84	9.59	7.28
4	73.20	7.30	7.25
7	81.94	4.61	6.72
10	83.08	3.70	6.61

5.3.3 Phosphoryl transfer from phosphoenolpyruvate to AMP or ADP in the presence of Fe(II).

Mixtures containing MES buffer (0.5M, pH 6.5) phosphoenolpyruvate (0.05M), AMP or ADP (0.05M) and ferrous sulfate (0.1M) were incubated at 45°C. Aliquots were removed at various times, treated with potassium cyanide, and analysed by ^{31}P NMR.

AMP

Time (day)	[AMP]/mM	[Pi]/mM	[PEP]/mM	[PPi]/mM
1	33.95	51.94	12.29	0.91
2	35.34	54.69	6.51	1.73
3	37.81	57.88	1.81	1.25
4	43.23	56.77	0.00	0.00

ADP

Time (day)	[AMP]/mM	[Pi]/mM	[PEP]/mM	[PPi]/mM	[ADP]/mM
1	7.19	51.77	19.29	2.99	32.91
2	10.41	62.87	11.07	5.66	27.33
3	15.51	61.01	4.82	7.91	26.43
4	18.63	63.06	2.39	4.74	28.50

5.3.4 Hydrolysis of 2-phosphoglycerate in the presence of Fe(II).

A mixture containing MES buffer (0.5M, pH 6.5), 2-phosphoglycerate (0.025M) and ferrous sulfate (0.1M) was incubated at 95°C. Aliquots were removed at various intervals, treated with potassium cyanide, and analysed by ^{31}P NMR.

Time (hr)	[Pi]/mM	[2PG]/mM
1	3.63	21.37
5	14.70	10.30
10	19.12	5.88
24	25.00	0.00

5.3.5 Hydrolysis of 3-phosphoglycerate in the presence of Fe(II).

A mixture containing MES buffer (0.5M, pH 6.5), 3-phosphoglycerate (0.25M) and ferrous sulfate (0.1M) was incubated at 95°C. Aliquots were removed at various intervals, treated with potassium cyanide, and analysed by ^{31}P NMR.

Time (day)	[Pi]/mM	[3PG]/mM
2	2.14	22.86
4	1.68	23.32
8	3.10	21.90

5.3.6 Hydrolysis of AMP in the presence of Fe(II).

A mixture containing MES buffer (0.5M, pH 6.5), AMP (0.025M) and ferrous sulfate (0.1M) was incubated at 95°C. Aliquots were removed at various intervals, treated with potassium cyanide, and analysed by ^{31}P NMR.

Time (day)	[Pi]/mM	[AMP]/mM
2	1.09	23.91
4	1.06	23.94
8	0.76	24.25

5.3.7 Attempted methylation of the reaction mixture remaining from the hydrolysis of 2-phosphoglycerate in the presence of Fe^{2+}.

The remaining reaction mixture(~1mL) was acidified with 2M HCl (2mL) and extracted with ether (3 x 2mL). Methylation reactions were performed on both the organic and the aqueous fractions.

A) Organic fraction.

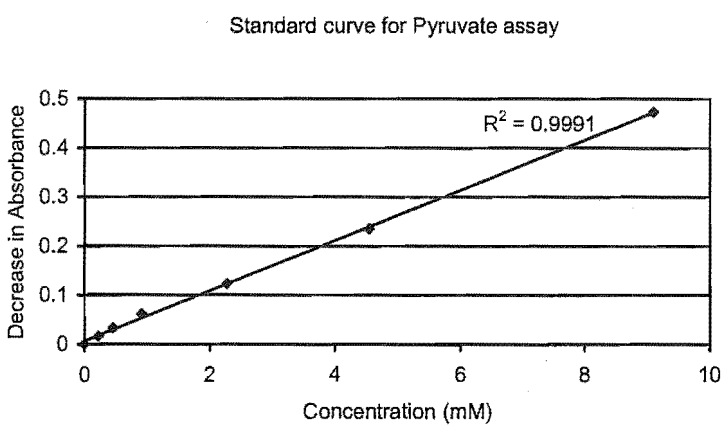
The ether was removed *in vacuo* and the residue was redissolved in methanol (2mL) and conc. sulfuric acid (1mL) and heated at 50°C for 30min. The methylation reaction was quenched by adding water (2mL), and extracted with ether. The ether was removed *in vacuo* and the residue analysed for methyl pyruvate by GC-MS.

B) Aqueous fraction.

The aqueous fraction was first concentrated under vacuum, then methanol (5mL) and conc. sulfuric acid (2mL) were added. The solution was heated at 50°C for 30min, and then quenched by the addition of water (5mL). This was extracted with ether and the organic phases combined. The solvent was removed *in vacuo* and the residue analysed for methyl pyruvate by GC-MS.

5.3.8 Enzyme assay for pyruvate.

The enzyme assay used was based on one used by Kraunsoe.[3] All the solutions were made up in 5mM Tris.HCl (pH 7.2). The reaction mixture used for the assay was prepared from the following solutions: NADH (20mL, 0.24mM), BSA (500μL, 1mg/mL), Tris.HCl (5mL, 5mM, pH 7.2) and L-lactate dehydrogenase (1mL, 55units/mL). 1mL of the mixture was pipetted into a cuvette and the absorbance measured at 340nm. 10μL of a solution containing pyruvate was then added and the decrease in the absorbance at 340nm was measured. A standard curve was constructed from a 0.91M solution of pyruvate that was systematically diluted and assayed in the above manner.



Least squares regression gave the following equation for the concentration of pyruvate in the 10μL aliquot:

$$[\text{Pyruvate}] \text{ in mM} = \frac{-\Delta Abs - 0.068}{0.0511}$$

5.3.9 Analysis for pyruvate from the hydrolysis of 2-phosphoglycerate in the presence of Fe^{2+}.

For this experiment the barium salt of 2-phosphoglycerate (>80% purity, Fluka) was used. This was converted into a solution of the sodium salt prior to use in the following manner. Sulfuric acid (2M, 0.1mL) was added to 2-phosphoglycerate (Ba^{2+} salt, 33.9mg) and vigorously mixed. Distilled water (0.3mL) was then added and the solution was centrifuged to remove the insoluble barium sulfate. The supernatant was removed and adjusted to neutral pH with sodium hydroxide (2M, 0.1mL). This solution was used in the following reaction.

A mixture containing MES buffer (0.5M, pH 6.5), 2-phosphoglycerate (~0.05M) and ferrous sulfate (0.1M) in a total volume of 2mL was incubated at 95°C. At various intervals aliquots of 50μL were removed and treated with potassium cyanide solution (1.2M, 50μL). 100μL of Tris.HCl (5mM, pH 7.2) was added to this and the sample was centrifuged. Aliquots (10μL) of the supernatant were then assayed for pyruvate as described above.

Time (min)	Average ΔAbs_{340}
0	0.0003
30	0.0063
60	0.0177
90	0.0083
120	0.0040
380	0.0157
1180	-0.0013

5.4 Experimental for Chapter 4: Polyphosphate Chemistry.

5.4.1 The hydrolysis of pyrophosphate.

A solution containing MES buffer (0.5M, pH 6.5) and pyrophosphate (0.025M) was heated at 95°C. 400μL aliquots were withdrawn at various intervals and frozen. Prior to analysis by ^{31}P NMR, a small amount of EDTA in 100μL D_2O was added to each sample.

Time (min)	[Pi]/mM	[PPi]/mM
15	1.60	24.21
30	6.45	21.78
60	21.91	14.05
120	41.98	4.01
180	46.42	1.79

5.4.2 The hydrolysis of pyrophosphate in the presence of amines.

A solution containing MES buffer (0.5M, pH 6.5) pyrophosphate (0.025M) and amine (0.05M) was heated at 95°C. 400μL aliquots were withdrawn at various intervals and frozen. Prior to analysis by ^{31}P NMR, a small amount of EDTA in 100μL D_2O was added to each sample.

Imidazole

Time (min)	[Pi]/mM	[PPi]/mM
15	1.55	24.23
30	4.53	22.74
60	18.61	16.82
120	33.92	8.04
180	44.44	2.78

Glycine.

Time (min)	[Pi]/mM	PPi]/mM
15	2.04	23.98
30	6.59	21.71
60	20.04	14.98
120	37.44	6.28
180	45.22	2.39

5.4.3 The hydrolysis of pyrophosphate in the presence of Fe(II).

A mixture containing MES buffer (0.5M, pH 6.5), pyrophosphate (0.025M) and ferrous sulfate (0.1M) was heated at 95°C. Aliquots were withdrawn at various intervals, treated with potassium cyanide, and analysed by ^{31}P NMR.

Time (hr)	[Pi]/mM	[PPi]/mM
1.5	7.85	21.08
3	13.40	18.30
4.5	17.56	16.22
6	20.88	14.56
23	39.96	5.02

5.4.4 The hydrolysis of pyrophosphate in the presence of Fe(II) and amines.

A mixture containing MES buffer (0.5M, pH 6.5), pyrophosphate (0.025M), imidazole (0.05M) and ferrous sulfate (0.1M) was heated at 95°C. Aliquots were withdrawn at various intervals, treated with potassium cyanide, and analysed by ^{31}P NMR.

Imidazole

Time (hr)	[Pi]/mM	[PPi]/mM
1.5	14.44	17.78
3	22.11	13.95
4.5	26.08	11.96
6	29.57	10.22
23	50.00	0.00

Glycine

Time (hr)	[Pi]/mM	[PPi]/mM
1.5	10.03	19.99
3	16.00	17.00
4.5	17.55	16.23
6	20.68	14.66
23	41.45	4.28

AMP

Time (hr)	[Pi]/mM	[PPi]/mM
0	0.00	25.00
1.5	4.31	22.85
3	6.80	21.60
4.5	7.21	21.40
11	10.16	19.92
23	20.58	14.71

5.4.5 Hydrolysis of pyrophosphate in the presence of Zn(II).

A mixture containing MES buffer (0.5M, pH 6.5), pyrophosphate (0.025M) and zinc sulfate (0.1M) was heated at 95°C. 400μL aliquots were withdrawn at various intervals, treated with potassium cyanide (~12eq CN^-/Zn^{2+}) in 100μL D_2O, and analysed by ^{31}P NMR

Time (min)	[Pi]/mM	[PPi]/mM
30	8.84	16.16
60	12.24	12.76
90	15.97	9.03
180	21.08	3.92
270	24.23	0.77

5.4.6 Hydrolysis of pyrophosphate in the presence of Ni(II).

A mixture containing MES buffer (0.5M, pH 6.5), pyrophosphate (0.025M) and nickel sulfate (0.1M) was heated at 95°C. 400μL aliquots were withdrawn at various intervals, treated with potassium cyanide (~12eq CN^-/Ni^{2+}) in 100μL D_2O, and analysed by ^{31}P NMR

Time (hr)	[Pi]/mM	[PPi]/mM
2	4.27	20.73
4	5.67	19.33
6	7.91	17.09
12	10.21	14.79
24	18.48	6.52

5.4.7 Hydrolysis of pyrophosphate in the presence of FeS.

A mixture containing MES buffer (0.5M, pH 6.5), pyrophosphate (0.025M), ferrous sulfate (0.1M) and sodium hydrogen sulfide (0.1M) was heated at 95°C. Aliquots were removed at various intervals, treated with potassium cyanide, and analysed by ^{31}P NMR.

Time (hr)	[Pi]/mM	[PPi]/mM
2	5.99	22.00
4.5	12.42	18.79
7.5	16.69	16.65
19	25.37	12.31

5.4.8 Hydrolysis of tripolyphosphate.

A solution containing MES buffer (0.5M, pH 6.5) and tripolyphosphate (0.025M) was heated at 95°C. 400μL aliquots were removed at various intervals, added to a small amount of EDTA in 100μL D_2O, and analysed by ^{31}P NMR.

Time (min)	[Pi]/mM	[PPi]/mM	[PPPi]/mM
0	0.00	3.66	21.34
15	2.94	8.20	19.18
30	8.79	9.91	15.47
60	25.88	13.39	7.43
90	39.92	12.86	3.12
120	48.00	11.12	1.59
240	65.65	4.68	0.00

5.4.9 Hydrolysis of tripolyphosphate in the presence of Fe(II).

A mixture containing MES buffer (0.5M, pH 6.5), tripolyphosphate (0.025M) and ferrous sulfate (0.1M) was heated at 95°C. Aliquots were removed at various intervals, treated with potassium cyanide, and analysed by ^{31}P NMR.

Time (min)	[Pi]/mM	[PPi]/mM	[PPPi]/mM
0	0.00	3.66	21.34
15	9.58	14.58	12.09
30	16.49	20.22	6.02
60	21.60	25.73	0.71
210	26.64	24.18	0.00
420	24.39	25.31	0.00
1230	27.32	23.84	0.00

5.4.10 Hydrolysis of ADP.

A solution containing MES buffer (0.5M, pH 6.5) and ADP (0.025M) was heated at 95°C. 400μL aliquots were removed at various intervals and added to a small amount of EDTA in 100μL D_2O before analysis by ^{31}P NMR.

Time (min)	[Pi]/mM	[AMP]/mM	[ADP]/mM
30	3.91	4.19	20.95
60	9.62	9.53	15.43
90	13.96	14.81	10.62
180	22.80	21.65	2.79
270	27.46	22.54	0.00
480	31.72	18.28	0.00

5.4.11 Hydrolysis of ADP in the presence of Fe(II).

A mixture containing MES buffer (0.5M, pH 6.5), ADP (0.025M) and ferrous sulfate (0.1M) was heated at 95°C. Aliquots were removed at various intervals, treated with potassium cyanide, and analysed by ^{31}P NMR.

Time (min)	[Pi]/mM	[AMP]/mM	[ADP]/mM
0	0.0	0.00	25.00
15	3.25	4.35	21.21
30	8.11	8.41	16.74
60	14.15	18.28	8.78
90	19.42	21.90	4.34

5.4.12 Hydrolysis of ATP.

A solution containing MES buffer (0.5M, pH 6.5) and ATP (0.025M) was heated at 95°C. 400μL aliquots were removed at various intervals and added to a small amount of EDTA in 100μL D_2O before analysis by ^{31}P NMR.

Time (min)	[Pi]/mM	[AMP]/mM	[ADP]/mM	[ATP]/mM
0	0.00	0.00	0.00	25.00
30	4.45	0.00	3.35	19.05
60	13.99	2.96	6.16	11.21
90	22.44	8.04	6.32	6.42
180	42.38	17.27	2.98	1.14
270	51.96	23.04	0.00	0.00
480	56.84	18.15	0.00	0.00

5.4.13 Hydrolysis of ATP in the presence of Fe(II).

A mixture containing MES buffer (0.5M, pH 6.5), ATP (0.025M) and ferrous sulfate (0.1M) was heated at 95°C. Aliquots were removed at various intervals, treated with potassium cyanide and analysed by ^{31}P NMR.

Time (min)	[Pi]/mM	[AMP]/mM	[PPi]/mM	[ADP]/mM	[ATP]/mM
0	0.00	0.00	0.00	0.00	25.00
15	12.16	2.60	0.75	10.90	11.58
30	18.30	7.17	5.25	11.39	6.67
60	28.01	16.90	6.74	6.65	1.11
90	32.66	24.46	6.99	1.76	0.00
360	40.91	29.11	2.49	0.00	0.00

5.4.14 Hydrolysis of ATP in the presence of FeS.

A mixture containing MES buffer (0.5M, pH 6.5), ATP (0.025M), ferrous sulfate (0.1M) and sodium hydrogen sulfide (0.1M) was heated at 95°C. Aliquots were removed at various intervals, treated with potassium cyanide, and analysed by ^{31}P NMR.

Time (min)	[Pi]/mM	[AMP]/mM	[PPi]/mM	[ADP]/mM	[ATP]/mM
0	0.00	0.00	0.00	0.00	25.00
15	9.80	2.89	2.48	9.60	12.71
30	17.10	8.12	4.78	11.67	5.62
60	26.36	19.28	7.07	7.61	0.00
360	41.05	27.23	3.36	0.00	0.00
660	44.03	25.76	2.61	0.00	0.00

5.4.15 Hydrolysis of pyrophosphate in the presence of glucose and Fe(II).

A mixture containing MES buffer (0.5M, pH 6.5), pyrophosphate (0.025M), glucose (0.025M) and ferrous sulfate (0.1M) was heated at 95°C. 400μL aliquots were

removed at various intervals, treated with potassium cyanide and analysed by ^{31}P NMR.

Time (hr)	[Pi]/mM	[PPi]/mM
1.5	6.38	21.81
3	11.75	19.12
4.5	12.74	18.63
11	26.86	11.57
23	40.63	4.82

5.4.16 Hydrolysis of pyrophosphate in the presence of adenosine and Fe(II).

A mixture containing MES buffer (0.5M, pH 6.5), pyrophosphate (0.025M), adenosine (0.05M) and ferrous sulfate (0.1M) was heated at 95°C. Aliquots were removed at various intervals, treated with potassium cyanide, and analysed by ^{31}P NMR.

Time (hr)	[Pi]/mM	[PPi]/mM
2	19.20	15.40
4	26.43	11.79
6	31.65	9.18
11	39.05	5.48

5.5 References for Chapter 5.

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